

### **REMARKS**

Claims 1-17 and 19-26 constitute the pending claims in the present application. Claims 1-13, 16, and 19-21 are withdrawn as being directed to a non-elected invention. Applicants will cancel such claims upon indication of allowable subject matter. Claims 14, 17, 22-24, and 26 have been amended. The amended claims are fully supported by the specification (e.g., page 4, lines 7-29). No new matter has been introduced. Applicants further submit that the amendments are made merely to expedite allowance of claims directed to most commercially relevant embodiments of the present invention. Applicants reserve the right to pursue claims of similar or differing scope in the future.

Applicants note with appreciation that the Preliminary Amendment filed on June 30, 2004 has been entered in full.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

#### **Election/Restriction**

The Examiner has acknowledged Applicants' election, with traverse, of Group IV (claims 14-15 and 17-18) in the Response filed on June 30, 2004. Applicants note with appreciation that the Examiner has grouped newly added claims 22-26 into elected Group IV.

#### **Drawings**

As the Examiner suggested, Applicants will submit new formal drawings when the application is allowed.

#### **Information Disclosure Statement**

As the Examiner requested, Applicants enclose herewith a courtesy copy of PTO 1449 filed with the Information Disclosure Statement on September 27, 2002.

#### **Claim rejections under 35 U.S.C. 112, first paragraph**

Claims 14-15, 17, and 22-26 are rejected under 35 U.S.C. 112, first paragraph, as allegedly failing to comply with the enablement requirement. Applicants traverse these rejections to the extent that they are maintained in light of the amended claims.

Specifically, the Examiner asserts that “[t]he instant claims are drawn to a method for identifying an antagonist or inhibitor of the activity of a polypeptide, or a fragment, derivative or ortholog thereof. These terms can read on as few as one or more amino acids . . . the specification provide no guidance as to what amino acids may be changed without causing a detrimental effect to the protein to be produced. Further, it is unpredictable as to which amino acids could be removed and which could be added” (Office Action, page 4, lines 1-15).

Applicants contend that the claims are enabled not only for the use of polypeptides encoded by the polypeptides encoded by the gene sequences provided in the disclosure (e.g., ygbB, yfhC, yacE, ychB, yejD, yrfI, yggJ, yjeE, yiaO, yrdC, yhbC, ygbP, ybeY, gcpE, kdtB, pfs, ycaJ, b1808, yeaA, yagF, b1983, yidD, yceG, and yjbC), but also for variants of these polypeptides (e.g., fragments, derivatives or orthologs).

Nevertheless, solely to expedite prosecution of claims directed to commercially relevant subject matter, Applicants have amended claim 14 to more particularly define the structure and function of the fragment, derivative or ortholog. As amended, claim 14 recites that “said fragment, derivative or ortholog is a functional equivalent of the wildtype polypeptide.” Applicants submit that one of skill in the art, at the time the application was filed, could readily have prepared and tested the fragment, derivative or ortholog (e.g., for its ability to functionally rescue the bacterial cell in which the corresponding wildtype gene is deleted) satisfying the parameters in the relevant assays without undue experimentation. Moreover, this functional limitation overcomes the Office Action’s concern over whether the fragment could be as small as a single amino acid. Applicants submit that at least several amino acids would be necessary to retain the function of the full-length sequence.

Applicants submit that the characteristics of the fragment, derivative or ortholog are well defined in the specification. For example, the specification teaches that “the term ‘fragment or derivative’ denotes any variant the amino acid or nucleotide sequence of which deviates in its primary structure, e.g., in sequence composition or in length as well as to analogue components.

For example, one or more amino acids of a polypeptide may be replaced in said fragment or derivative as long as the modified polypeptides remain functionally equivalent to their described counterparts” (page 4, lines 7-11). The specification further describes that “[t]he term ‘orthologs’ as used herein means homologous sequences in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution” (page 4, lines 17-19).

Further, Applicants respectfully point out that one of skill in the art could practice the present invention without necessarily knowing which amino acid substitutions, deletions, or insertions to make, since the fragment, derivative or ortholog of amended claim 14 is clearly defined structurally and functionally. Applicants submit that the techniques of combinatorial mutagenesis (Reidhaar-Olson and Sauer 1988, enclosed herewith as **Exhibit A**) and high through-put screening, known in the art at the time of filing, make the identification of variant polypeptides (e.g., fragments, derivatives or orthologs) routine, if not trivial. The fields of combinatorial and scanning mutagenesis had trivialized the once complex and painstaking process of making and testing polypeptide variants long before the filing of the present application. These techniques were routinely practiced, and allow a wide range of amino acid substitutions to be made and tested for the maintenance or disruption of functional properties without undue experimentation. Accordingly, techniques for synthesizing, testing, and identifying sequences which would possess the claimed structures and functions were well known in the art at the time of filing and routinely carried out. One of skill in the art faced with the task of constructing the variant polypeptides within the scope of the claims at the time this application was filed would not have approached the problem by trial and error. Instead, the skilled artisan would have used combinatorial mutagenesis without undue experimentation.

The Examiner has cited three references (Rudikoff et al., Burgess et al., and Lazar et al.) to show that minor changes in an amino acid sequence may dramatically change a protein function. Although Applicants agree that it is possible to abolish activity of a given protein by changing a critical residue as disclosed by these cited reference, Applicants disagree that this fact means that a skilled artisan cannot make functional variants (e.g., fragments, derivatives or orthologs) of a protein without undue experimentation. In fact, Rudikoff et al. teach that “as many as eight or nine substitutions may occur in hypervariable regions with no significant effect

on hapten affinity or specificity” (page 1982, column 2, lines 1-2). Based on the teachings of Rudikoff et al., one can expect that substitutions in a given protein can produce variant proteins with normal or nearly normal activity.

Applicants further point out that the making of polypeptide variants was and is routinely practiced in the art using common and routine laboratory techniques. One of skill in the art would know that conservative substitutions in an amino acid sequence would be likely to avoid significant changes in its activity. These substitutions can be made based on both the sequence data and on knowledge of the structure of the twenty amino acids. Amino acid substitutions can be chosen in order to maintain or disrupt the shape and charge density of a region of the protein, and one of skill in the art would have recognized that whether a particular amino acid substitution would maintain or disrupt a given region of a protein is highly predictable based on the structure of the twenty natural amino acids. For example, arginine and lysine are positively charged amino acids and the substitution of one for the other is routinely practiced in the art without significantly affecting the function of the overall sequence.

In addition, Applicants point out that even if the claims encompass certain inoperative embodiments, that does not undermine the enablement of the claims as a whole. In accordance with MPEP 2164.08(b), “[t]he presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is normally required in the art.” This standard has been upheld in the courts, and permits a claim to encompass a finite number of inoperable embodiments so long as inoperable embodiments can be determined using methodology specified in the application without undue experimentation. See, for instance, *In re Angstadt*, 190 U.S.P.Q. 214 (CCPA 1976).

In view of the arguments and amendments presented above, Applicants submit that all pending claims as amended fully comply with the enablement requirement. Applicants assert that the cited references are rendered irrelevant in view of the claim amendments. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. 112, first paragraph, is respectfully requested.

Claim rejections under 35 U.S.C. 112, second paragraph

Claims 14-15, 17, and 22-26 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully traverse this rejection to the extent it is maintained over the claims as amended.

First, the phrase “fragments, derivatives or analogues thereof” in claims 14 and 17 is objected to as allegedly being vague and indefinite. After pointing out that the term “analogues” does not appear in the claims – it is “ortholog” instead, a term that has a very particular and well understood meaning – Applicants respectfully submit that these terms are amply supported by the specification. Furthermore, as described above, the present amendment recites that the fragment be functionally equivalent to the full-length sequence, effectively eliminating the possibility that the fragment could be as small as one amino acid; such short sequences would not retain the functional activity of the full-length sequence.

As described above, the specification states that “the term ‘fragment or derivative’ denotes any variant the amino acid or nucleotide sequence of which deviates in its primary structure, e.g., in sequence composition or in length as well as to analogue components. For example, one or more amino acids of a polypeptide may be replaced in said fragment or derivative as long as the modified polypeptides remain functionally equivalent to their described counterparts. The term ‘fragment or derivative’ further denotes compounds analog to an antagonist or inhibitor that should have a stabilized electronic configuration and molecular conformation that allows key functional groups to be presented to the mentioned polypeptide in substantially the same way as the antagonist and inhibitor. The variant of the polypeptide may be a naturally occurring allelic variant of the polypeptide or non-naturally occurring variants of those polynucleotides.” (page 4, lines 7-16).

The specification further describes that “[t]he term ‘orthologs’ as used herein means homologous sequences in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. However, orthologous genes may or may not be responsible for a similar function (see, e.g., the glossary of the ‘Trends Guide to Bioinformatics’, Trends Supplement 1998, Elsevier Science). Orthologous

genes, nucleic acids or proteins comprise genes, nucleic acids or proteins which have one or more sequences or structural motifs in common.” (page 4, lines 17-23). In addition, Applicants point out that all genes of the application were selected on the basis of conservation between twelve bacterial species, providing further support for the term “ortholog” in the claims.

In view of the above descriptions in the specification, one of skill in the art would readily understand the term “fragment, derivative or ortholog thereof” in the pending claims.

Second, the phrase “interaction of said polypeptide molecule with polypeptide” in claims 22-23, 24, and 26 is objected to as allegedly being vague and indefinite. Applicants have amended claim 17 to recite a “molecule” instead of a “polypeptide molecule,” and further specify that “wherein said molecule is a second polypeptide.” Dependent claims 22-23, 24, and 26 have been correspondingly amended to recite “said molecule.” Applicants submit that claim 17 makes it clear that the first polypeptide is encoded by a gene selected from ygbB, yfhC, yacE, ychB, yejD, yrfI, yggJ, yjeE, yiaO, yrdC, yhbC, ygbP, ybeY, gcpE, kdtB, pfs, ycaJ, b1808, yeaA, yagF, b1983, yidD, yceG, or yjbC. Therefore, these amended claims are definite and clear to one skilled in the art.

Third, the phrase “small molecule” in claim 25 is objected to as allegedly being vague and indefinite. Applicants submit the term “small molecule” is a term of art whose meaning was understood by those skilled in the art. A review of the literature prior to the priority date reveals that it was well accepted that small molecules are organic molecules with a molecular weight of less than about 1000 daltons. The term is used to differentiate these organic molecules from typical large biomolecules like nucleic acids, proteins, and complex carbohydrates like heparin and starch. In support of this understanding, Applicants respectfully direct the Examiner’s attention to the following exemplary citations:

(1) Free Radical Toxicology (Target Organ Toxicology Series) by Kendall B. Wallace, Publisher: Taylor & Francis; (June 1997), ISBN: 1560326328, which states on page 148 “... is associated with three chemically distinct types of oxidants formed by iron-mediated Fenton reactions in the presence of DNA. Small-Molecule Antioxidants Numerous **small molecules (<1000 MW)** with high reactivity toward oxidants have been described. Three of these, vitamin E, ascorbic acid, and glutathione, play essential ...” [emphasis added]

(2) Molecular Methods for Virus Detection by Danny Wiedbrauk and Daniel Farkas, Publisher: Academic Press, 1st edition (January 15, 1995), ISBN: 0127489207, which states on page 154 "Electrochemiluminescent labels are relatively **small molecules (--1000 dalton)** that are extremely stable and may be coupled to nucleic acids, haptens, or proteins without affecting immunoreactivity or ..." [emphasis added]

(3) Neurotoxicology: In Vitro by V. W. Pentreath (Editor), Publisher: Taylor & Francis; 1 edition (June 1, 1999), ISBN: 0748403884, which states on page 200 "... Modalities of intercellular communication. Hormones (V) and growth factors (0) are transported to the targets via blood. Ions and **small molecules (< 1000 Da)** can pass through gap junctions from one cell to its neighbours and influence the function of the connecting cells. ..." [emphasis added]

(4) New Frontiers in Cancer Causation: Proceedings of the Second International Conference on Theories of Carcinogenesis, by Olav Hilmar Iversen (Editor), Publisher: Taylor & Francis, (September 1993), ISBN: 1560322519, which states on page 186 "... (connexons). Each cell contributes a hemichannel composed of a hexamer of proteins (connexins). Clusters of these connexons allow ions and **small molecules (below 1000 daltons)** to freely equilibrate between coupled cells. There exists a family of highly conserved genes coding for these proteins ..." [emphasis added]

(5) Dermatotoxicology by Francis Nicholas Marzulli (Editor), Howard I. Maibach (Editor), Publisher: Taylor & Francis; 5th edition (February 1996), ISBN: 1560323566, which states on Page 147 "... with skin proteins to form complete antigens and how these structures are recognized by T-cell receptors. SOME CHEMICAL REMINDERS Haptens (**small molecules with a molecular mass less than 1000 Da**) interact with biological macromolecules by mechanisms leading to the formation of bonds of various strengths between the two entities. ..." [emphasis added]

Copies of these articles are provided as **Exhibit B**. In addition, Applicants provide herewith a copy of a PubMed printout for a search of the term "small molecule". An exemplary page of twenty results roughly contemporaneous with the filing of the priority document is provided as **Exhibit C**, together with the abstracts of the last ten of these articles. All of these references use the term consistently with the above references, i.e., to refer to low molecular weight organic compounds, and the particular small molecules singled out by these references are all consistent with the understanding set forth above. Moreover, these articles use the term in the abstract and/or title – where it is critical that the matter being described be clear, simple and well understood. It would be unfathomable to use a term in the title or abstract that would invite

confusion or misunderstanding among those of ordinary skill in the art. And hundreds of references, as can readily be seen, used the term "small molecule" in a similar manner well before the filing of the present application. Accordingly, Applicants submit that the citations taken together are indicative of the well accepted meaning of small molecules or small organic compounds, and that the meaning of such terms would be understood by one skilled in the art. In re Hammack, 166 USPQ 204, 208 (C.C.P.A 1970).

In view of the above amendments and remarks, all pending claims have satisfied the requirements under 35 U.S.C. § 112, second paragraph. Reconsideration and withdrawal of the rejections are respectfully requested.

### **CONCLUSION**

For the foregoing reasons, Applicants respectfully request reconsideration and withdrawal of the pending rejections. Applicants believe that the claims are now in condition for allowance and early notification to this effect is earnestly solicited. Any questions arising from this submission may be directed to the undersigned at (617) 951-7000.

If there are any other fees due in connection with the filing of this submission, please charge the fees to our **Deposit Account No. 18-1945**. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit account.

Respectfully Submitted,

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# Combinatorial Cassette Mutagenesis as a Probe of the Informational Content of Protein Sequences

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A method of combinatorial cassette mutagenesis was designed to readily determine the informational content of individual residues in protein sequences. The technique consists of simultaneously randomizing two or three positions by oligonucleotide cassette mutagenesis, selecting for functional protein, and then sequencing to determine the spectrum of allowable substitutions at each position. Repeated application of this method to the dimer interface of the DNA-binding domain of  $\lambda$  repressor reveals that the number and type of substitutions allowed at each position are extremely variable. At some positions only one or two residues are functionally acceptable; at other positions a wide range of residues and residue types are tolerated. The number of substitutions allowed at each position roughly correlates with the solvent accessibility of the wild-type side chain.

IT HAS BEEN MORE THAN 20 YEARS SINCE ANFINSSEN AND HIS colleagues showed that the sequence of a protein contains all of the information necessary to specify the three-dimensional structure (1). However, the general problem of predicting protein structure from sequence remains unsolved. Part of the difficulty may stem from the complexity of protein structures. Although some 200 protein structures are known, no rules have emerged that allow structure to be related to sequence in any simple fashion (2). The problem is further complicated by the nonuniformity of the structural information encoded in protein sequences. Some residue positions are important, and changes at these positions can tip the balance between folding and unfolding (3–7). Other residues are relatively unimportant in a structural sense and a wide range of substitutions or modifications can be tolerated at these positions (3, 7–9).

If only a fraction of the residues in a protein sequence contribute significantly to the stability of the folded structure, then it becomes important to be able to identify these residues. We now describe the results of genetic studies that allow the importance of individual residues in protein sequences to be rapidly determined. Specifically, we determine the spectrum of functionally acceptable substitutions at residue positions near the dimer interface of the  $\text{NH}_2$ -terminal domain of phage lambda ( $\lambda$ ) repressor (10). The  $\text{NH}_2$ -terminal domain binds to operator DNA as a dimer, with dimerization

mediated by hydrophobic packing of  $\alpha$  helix 5 of one monomer against  $\alpha$  helix 5' of the other monomer (11) (Fig. 1, A and B). Without helix 5 there are no contacts between the subunits (Fig. 1C). By applying combinatorial cassette mutagenesis to the helix 5 region, we find that the number and spectrum of allowable substitutions within helix 5 are extremely variable from residue to residue. In most cases, this variability can be rationalized in terms of the fractional solvent accessibility of the wild-type side chain.

**General strategy.** For our studies, we used a plasmid-borne gene that encodes a functional, operator-binding fragment (residues 1–102) of  $\lambda$  repressor (12). The binding of the 1–102 fragment to operator DNA depends on dimerization which, in turn, depends on the helix 5–helix 5' packing interactions (11, 13). Thus, if a 1–102 protein retains normal operator-binding properties, we can infer that it is able to dimerize normally.

Mutagenesis of the helix 5 region was performed by a combinatorial cassette procedure. One example of this method, in which codons 85 and 88 are mutagenized, is illustrated in Fig. 2. On the top strand, the mutagenized codons are synthesized with equal mixtures of all four bases in the first two codon positions and an equal mixture of G and C in the third position. The resulting population of base combinations will include codons for each of the 20 naturally occurring amino acids at each of the mutagenized residue positions. On the bottom strand, inosine is inserted at each randomized position because it is able to pair with each of the four conventional bases (14). The two strands are then annealed and the mutagenic cassette is ligated into a purified plasmid backbone.

To identify plasmids encoding functional protein, we selected transformants for plasmid-encoded resistance to ampicillin and for resistance to killing by  $cI^-$  derivatives of phage  $\lambda$ . The latter selection requires that the cell express 1–102 protein that is active in operator binding (15). For each mutagenesis experiment, many independent transformants were chosen, single-stranded plasmid DNA was purified, and the relevant region of the 1–102 gene was sequenced. The resulting set of sequences provides a list of functionally acceptable helix 5 residues.

**Substitutions in the helix 5 region.** In separate experiments with different mutagenic cassettes, the codons for helix 5 residues 85 and 88; 86 and 89; 90 and 91; 84, 87, and 88; and 84, 87, and 91 were mutagenized, and genes encoding active 1–102 proteins were selected. In some cases, the survival frequency was low. For example, only 17 of 60,000 transformants passed the selection after randomization of codons 84, 87, and 88. In this case, each active candidate was sequenced. By contrast, 1,200 of 50,000 transformants passed the selection in the mutagenesis of positions 86 and 89 (16). In this case, we picked 50 candidates for sequence analysis. Overall, 150 active genes were sequenced (Table 1). In addition, we sequenced

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From the list of allowed substitutions, several conclusions may be

Positions 84 and 87 in particular stand out as having a high informational content. Ile appears to be the only acceptable residue at position 84. Both Met and Leu are residues of similar size and hydrophobicity, and are the only two residues that appear to be functional at position 87. The side chains of Ile<sup>84</sup> and Met<sup>87</sup> form a major part of the helix-helix packing interaction at the dimer interface, where Ile<sup>84</sup> of one subunit packs against Met<sup>87</sup> of the other subunit, and vice versa (Fig. 4). This cluster of four residues also contacts the globular portions of the domain. Solvent accessibility calculations by the method of Lee and Richards (17) show that the Ile<sup>84</sup> and Met<sup>87</sup> side chains are almost completely buried (92 to 98 percent solvent inaccessible) in the structure of the dimer. We assume that replacement of Ile<sup>84</sup> or Met<sup>87</sup> with smaller side chains would diminish dimerization because hydrophobic and van der Waals interactions would be lost. In fact, mutant repressors containing Ser<sup>84</sup> or Thr<sup>87</sup> are defective in dimerization (13, 18). Replacing Ile<sup>84</sup> or Met<sup>87</sup> with larger residues would also be expected to be detrimental because substantial structural rearrangements would be required to accommodate larger side chains.

Seven residues (Leu, Ile, Val, Thr, Cys, Ser, and Ala) are functionally acceptable at position 91. Aromatic residues, charged residues, and strongly hydrophilic residues are not found. The wild-type Val side chain is partially buried in the dimer structure, with the Cy2 methyl group packing against the C81 methyl group of the Ile<sup>84'</sup> side chain. Although some of the acceptable substitutions such as Ile and Thr could make equivalent packing contacts, others such as Ala and Ser could not.

Nine residues (Trp, His, Met, Gln, Leu, Val, Ser, Gly, and Ala) are acceptable at position 90. There is a surprisingly large range in both the acceptable size and hydrophobicity of these side chains. This is especially true as the C $\beta$  methyl group of the wild-type Ala is almost completely buried in the structure of the dimer and, at first glance, it would appear that larger side chains could not be accommodated. However, the inaccessibility of the C $\beta$  methyl group of Ala<sup>90</sup> is largely caused by the Lys<sup>67</sup> side chain, which packs against it. By rotating the Lys<sup>67</sup> side chain away, we were able to introduce a Trp<sup>90</sup> side chain by model-building without steric clashes. Rotation of the Lys<sup>67</sup> side chain away from Ala<sup>90</sup> should not be energetically costly and, in fact, is observed in crystals of the NH<sub>2</sub>-terminal domain bound to operator DNA (19).

Nine different residues (Trp, Tyr, Phe, Met, Ile, Val, Cys, Ser, and Ala) are functionally acceptable at position 88. There are large variations in the sizes and volumes of the acceptable side chains, although most are relatively hydrophobic. Charged residues and other strongly hydrophilic residues are not observed. In the wild-type dimer (11), the aromatic ring of Tyr<sup>88</sup> stacks against the ring of Tyr<sup>88'</sup>. The side chains of Trp, Phe, Met, Ile, and Val could probably form some type of packing interaction at this position, although those of Ala and Ser could not. It is known that the presence of Cys at position 88 allows a stable Cys<sup>88</sup>-Cys<sup>88'</sup> disulfide bond, which links the monomers in a conformation that is active in operator binding (20).

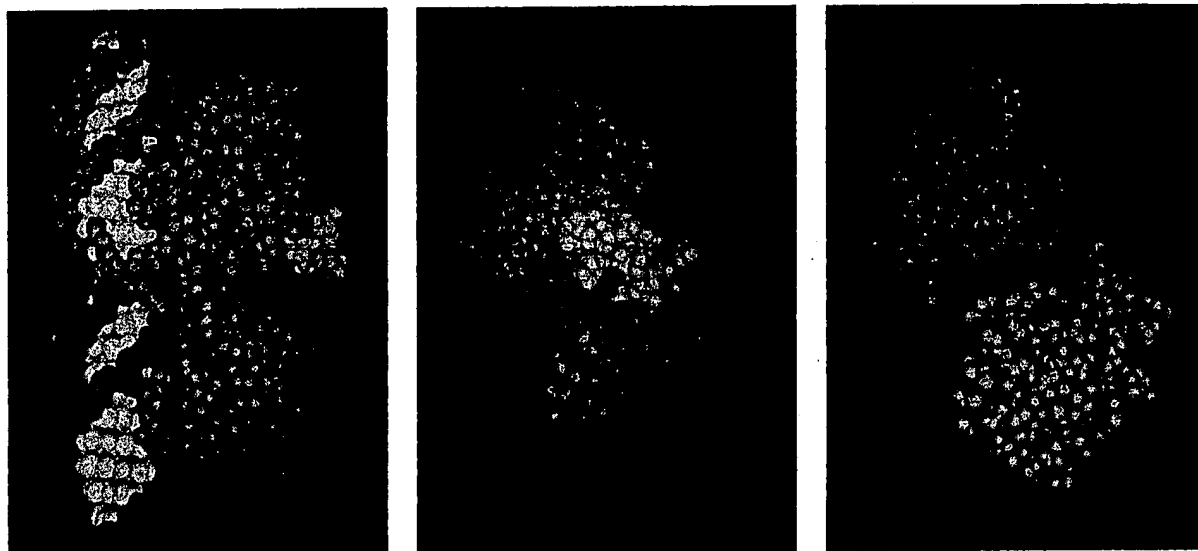
Positions 85, 86, and 89 show considerable variability. At each of these positions, 13 different amino acids were found to function. At positions 85 and 86, aromatic, hydrophobic, polar, and charged residues are all acceptable. At position 89, aromatic residues were not represented, but each of the remaining classes was observed. In

Active

85	90		85	90		85	90		85	90	
IYEMYEAV			-L-V---			--Y-S-			--E-L*		
I--MF---	2		-L-M---			--Y-T-			--E-E-		
I--MY---	4		-I-F---			--W-A-			--E-G*		
I-AMA---			-I-A---			--W-C-			--G-E-		
I-DMY---			-I-S---			--W-Q-					
I--MA---	3		-Q-Y---			--W-S-	3		IYEMYEAV		
I--MI---			-S-Y---			--W-K*			-----WA	4	
I--LF---			-S-W---			--W-D-			-----WL*		
I--LW---			-T-Y---			--W-D*			-----WS		
			-T-W---			--W-E-			-----HV		
IYEMYEAV			-T-A---			--W-G-	3		-----AV	6	
I--M---V			-R-F---			-A-E-	3		-----AC	2	
I--M---T			-R-W---	4		-SM-A-			-----AL	4	
I--L---T			-R-A---	3		-M-S-	3		-----AI		
			-R-C---			-L-S-	2		-----AT	2	
			-E-F---			-L-T-			-----VA		
IYEMYEAV			-G-Y---			--L-E-			-----VC	2	
-Y-F---						--L-E*			-----MA		
-W-W---	2		IYEMYEAV			--I-S-			-----LA*		
-W-A---			--F-M-			--Q-S-			-----QV		
-A-Y---			--F-L-			--S-Q-			-----QT		
-V-Y---	2		--F-I-			--S-E-			-----SV	3	
-V-A---	3		-F-Q---			-T-E-			-----SC	2	
-C-F---	2		--F-S-	3		--D-L-			-----SL	5	
-C-A---			--F-T-			--D-Q-			-----ST		
-L-F---			-F-R---			--D-D*			-----GC		
-L-W---			-F-E---			--E-L-	2		-----GI		
-L-A---									-----CM		

*Inactive*

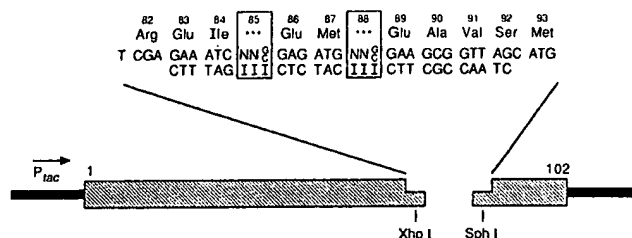
85	90	85	90	85	90	85	90
A--VA---	*	P--DS---	*	Q--CS---	*	-----RN*	
P--PL---	*	R--TR---	*	A--TP---	*	-----EA*	
P--TN---	*	T--TV---	*	S--TK---	*	-----KV*	
R--NP---	*	R--VI---	*	T--LN---	*	-----VM*	
P--LL---	*	L--PL---	*	A--SL---	*	-----PA*	
A--IL---	*	I--LL---	*	R--WS---	*	-----NQ*	
T--KP---	*	K--IV---	*	-----PR*		-----ME*	
Q--RV---	*	C--YT---	*	-----PP*5		-----AY*	
H--DVR---	*						



**Fig. 1.** Three views of the DNA-binding domain of  $\lambda$  repressor, showing the role of helix 5 in dimerization. (A) Proposed complex of repressor dimer with operator DNA (11). Helix 5 of each monomer is colored more lightly than the globular portion of that monomer. (B) Free repressor dimer,

rotated 90° from the view in (A), to show the "back side" of the molecule. (C) Dimer with helix 5 of each monomer removed. This view illustrates the role helix 5 plays in mediating dimerization (26).

**Fig. 2.** Schematic diagram showing the combinatorial cassette mutagenesis procedure. At positions indicated as N, an equal mixture of A, G, C, and T was used during oligonucleotide synthesis. At positions indicated as I, inosine was used. After synthesis, the oligonucleotides were phosphorylated, annealed, and ligated into the Xho I–Sph I backbone of plasmid pJO103. Plasmid pJO103 is an M13 origin plasmid with the 1–102 gene under control of a *tac* promoter; the region of the 1–102 gene encoding residues 82–93 (the small Xho I–Sph I fragment) is replaced by an unrelated 1.9-kb Xho I–Sph I "stuffer" fragment. Ligated DNA was transformed into *Escherichia coli* strain X90 F' *lacI*<sup>Q</sup> cells (27), and ampicillin-resistant colonies were selected in the presence or absence of phage  $\lambda$ KH54. Candidates that survived the selection were cross-streaked against a series of virulent derivatives of phage  $\lambda$  to confirm their immunity properties [strains and methods are described in (21)]. Single-stranded plasmid DNA was purified from an M13RV1 transducing lysate as described (28), and DNA sequences were determined by the dideoxy method (29).



the wild-type dimer, the side chains of Tyr<sup>85</sup>, Glu<sup>86</sup>, and Glu<sup>89</sup> are relatively solvent accessible.

Several amino acids are significantly underrepresented among the active sequences. For example, Pro is never found. This cannot be an artifact of our mutagenesis procedure because Pro is frequently observed among the unselected mutant sequences (Table 1). We conclude that Pro is not found among the functional sequences because it is selected against; its presence would presumably disrupt the  $\alpha$ -helical structure and thereby the helix-helix packing at the dimer interface.

His, Asn, and Lys are also underrepresented among the functional helix 5 sequences. These residues are presumably not acceptable at positions 84 and 87, where the informational content is extremely high, and may not be acceptable at positions 88 and 91, where the functional substitutions are generally hydrophobic in character. The acceptability of these residues at positions such as 85 and 86 is difficult to assess from our experiments because the codons for these residues are present at reasonably low frequencies even among the unselected sequences. In these cases, we probably have not sequenced a large enough number of candidates to be confident that all acceptable substitutions have been identified. In fact, data from reversion studies (21) and suppressed amber studies (22) show that His<sup>85</sup> and Lys<sup>86</sup> are acceptable substitutions in the context of the intact  $\lambda$  repressor molecule.

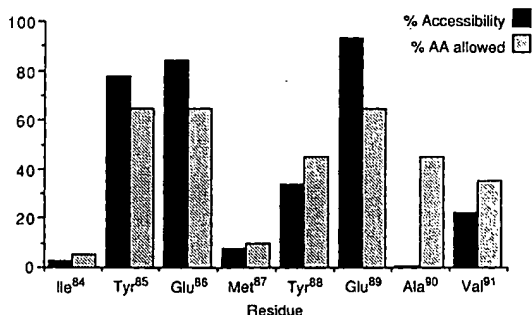
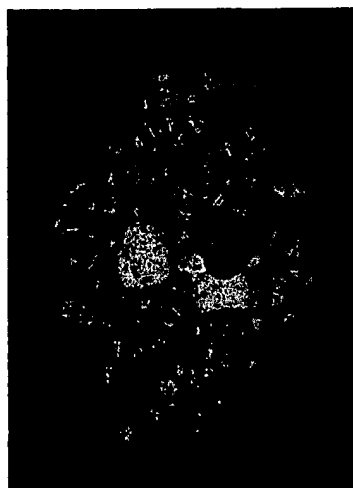
**Informational content and protein structure.** We have com-

Arg	Asp	Arg
Gln	Gln	Lys
Glu	Glu	Asp
Ser	Ser	Gln
Thr	Thr	Glu
Tyr	Tyr	Ser
Cys	Gly	Thr
Gly	Ala	Cys
Ala	Met	Gly
Trp	Trp	Ala
Leu	Leu	Met
Val	Phe	Trp
Ile	Ile	Leu
	Met	Ile
	Leu	Val
		Val
		Ile
Ile	Tyr	Glu
84	85	86
		87
		88
		89
		90
		91

**Fig. 3.** Functionally acceptable residues in the helix 5 region. The amino acids are listed from top to bottom in order of increasing hydrophobicity according to the scale of Eisenberg *et al.* (30).

bined an efficient combinatorial mutagenesis procedure and a functional selection to probe the informational content of the eight residues that form the major part of the dimerization interface of the NH<sub>2</sub>-terminal, operator-binding domain of  $\lambda$  repressor. At two of these eight residue positions, the functionally acceptable choices are highly restricted. For example, we analyzed 17 functional genes in which codon 84 had been randomized and recovered the wild-type residue, Ile, in every case. This is clearly a position of high

**Fig. 4.** Helix 5 residues high in informational content. The two isolated helix 5 regions of the protein are shown in green and blue. Ile<sup>84</sup> and Met<sup>87</sup> from the green helix are shown in yellow; Ile<sup>84</sup> and Met<sup>87</sup> from the blue helix are shown in red.



**Fig. 5.** Correlation between the solvent accessibility and the number of functionally acceptable substitutions. Hatched bars indicate the percentage of the 20 naturally occurring amino acids that are functionally acceptable at a residue position. Black bars indicate the fractional solvent accessibility of the wild-type side chain in the dimer. Solvent accessibilities for the NH<sub>2</sub>-terminal domain dimer (11) were computed using a 1.4 Å probe by the method of Lee and Richards (17). Fractional accessibilities were obtained by dividing by the appropriate side chain accessibilities calculated for the monomer. The fractional accessibilities change only slightly if the side chain accessibilities in the reference tripeptide Ala-X-Ala (17) are used instead as the reference state.

informational content. The informational content is also high at position 87, where Met and Leu are the only acceptable residues. By contrast, the remaining positions have moderate to low informational contents. For example, among 38 functional genes in which codon 85 had been randomized, the wild-type residue was recovered only once, and 12 other residues, differing in size and chemical properties, were recovered in the remaining cases. This is clearly a position of low informational content. It is striking that most of the structural determinants of dimerization in this eight-residue segment reside in two residues only. The remaining positions are surprisingly tolerant of a wide range of substitutions. If this high level of tolerance is generally true of protein sequences, then the problem of understanding and predicting structure may rest largely on the ability to identify those few residues that are crucial.

The positional variability of the informational content in helix 5 can, in general, be rationalized in terms of the solvent accessibility of the wild-type residues in the crystal structure (11). There is a rough correlation between the number of acceptable substitutions and the fractional extent to which the wild-type side chain is solvent accessible (Fig. 5). At exposed surface positions such as 85, 86, and 89, we find that many different residues and residue types can be functionally accommodated. By contrast, at positions such as 84 and

87, where the wild-type side chain is almost completely buried, we find that the functionally acceptable residue choices are extremely restricted. There is one apparent exception to the simple rule that buried residues are high in informational content. Ala<sup>90</sup> is inaccessible to solvent in the crystal structure, and yet we find that many substitutions are allowed at this position. However, the inaccessibility of the Ala<sup>90</sup> side chain to solvent is not due to close packing at the dimer interface, but rather to an interaction with a nearby surface side chain. This side chain can presumably move to allow larger side chains to be accommodated at position 90. Examples of this type demonstrate the need to distinguish between two types of buried side chains: those that can become exposed by relatively minor rearrangement of other side chains, and those that are tightly packed in the hydrophobic core.

There is no reason to assume that there should always be a strict correlation between the solvent accessibility of a residue and the structural informational content of that position. For one thing, the chemical properties of the 20 amino acids are not related in any simple linear fashion. Moreover, the structural importance of some residues in proteins almost certainly stems from interactions other than simple hydrophobic packing. Nevertheless, the closely packed nature of protein interiors (23) provides a simple molecular explanation for the structural importance of buried residues, and destabilizing mutations are commonly found to affect hydrophobic core residues (3–7). By contrast, missense mutations or chemical modifications that affect surface residues are often found to have little or no influence on protein stability (3, 7, 8). Thus, it is reasonable that solvent accessibility should be an extremely important determinant of the informational content of a residue position.

Our overall strategy for rapidly probing informational content should be broadly applicable to a wide range of protein structure-function problems in systems where genetic selections or screens can be devised. The method consists of three basic elements: (i) the use of cassette mutagenesis to introduce extremely high levels of targeted random mutagenesis; (ii) the use of a functional selection to identify genes encoding active proteins; and (iii) the use of rapid DNA sequencing methods to determine the spectrum of functionally acceptable residues in a relatively large number of candidates. Our method of combinatorial cassette mutagenesis (Fig. 2) allows several residue positions to be mutagenized at the same time and, in principle, generates a mutant population in which each of the 20 amino acids is represented at each mutagenized position (24). When two or three codons are mutagenized at the same time, the entire analysis is able to proceed more rapidly. Moreover, at this level of mutagenesis most two-residue and three-residue combinations should be present in the mutagenized population and should be recovered if they result in a functional protein. In our study of the packing of the 84 and 87 side chains, we recovered only two (Ile<sup>84</sup> with Met<sup>87</sup> and Ile<sup>84</sup> with Leu<sup>87</sup>) of the 400 possible residue combinations. Thus, because both positions were mutagenized in the same experiment, we are able to conclude that there are not significantly different ways of packing the dimer interface.

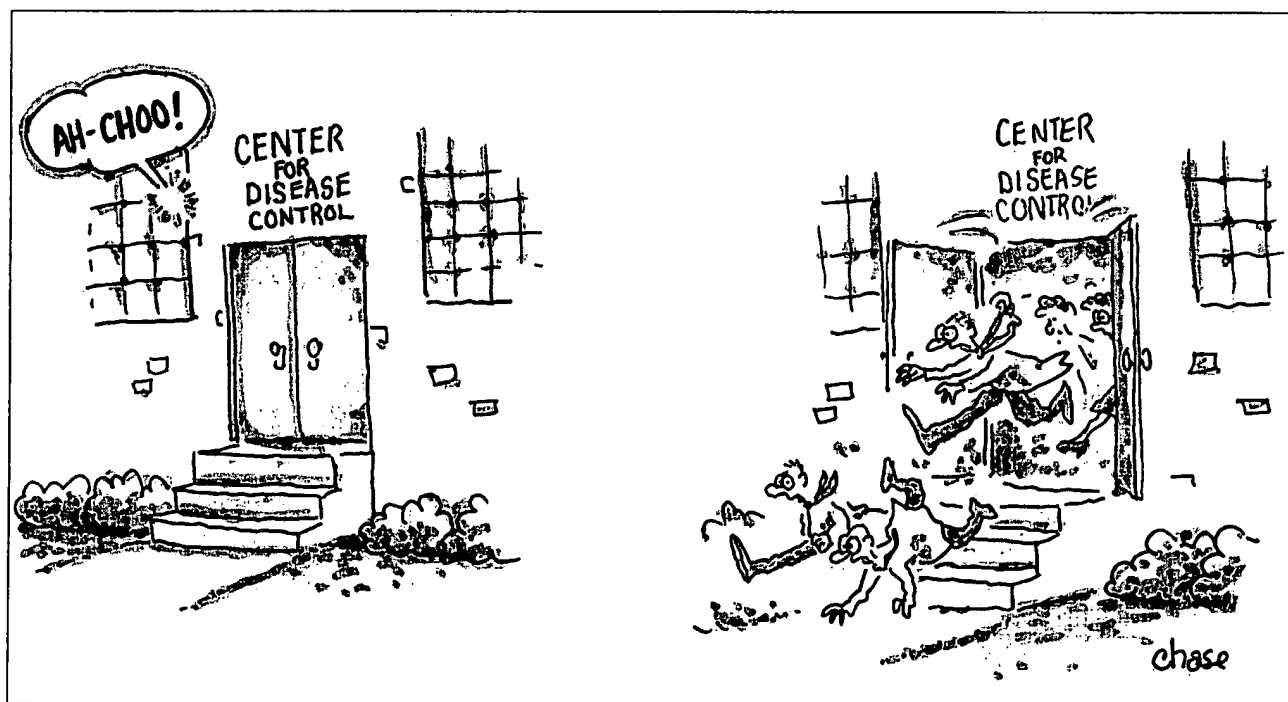
In principle, data like that shown in Fig. 3 could be generated for an entire protein sequence, and additional experiments could be devised to determine whether the positions of high informational content were important for structure or function. For proteins of unknown structure, such data might be quite useful for structural predictions. First, current predictive algorithms could be applied to the family of related sequences generated by our method, as each of these sequences is able to form the same basic structure. Second, because of their fundamental repeats,  $\alpha$ -helical and  $\beta$ -strand regions might be recognized by characteristic patterns of high and low informational content. Third, the positions of highest structural informational content should include the residues involved in

formation of the hydrophobic core of the protein. This information might prove useful in combination with the tertiary template ideas recently proposed (25).

#### REFERENCES AND NOTES

1. C. J. Epstein, R. F. Goldberger, C. B. Anfinsen, *Cold Spring Harbor Symp. Quant. Biol.* 28, 439 (1963); C. B. Anfinsen, *Science* 181, 223 (1973).
2. T. E. Creighton, *Proteins: Structures and Molecular Properties* (Freeman, New York, 1983), chap. 6.
3. M. H. Hecht, H. C. M. Nelson, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* 80, 2676 (1983); M. H. Hecht, J. M. Sturtevant, R. T. Sauer, *ibid.* 81, 5685 (1984); M. H. Hecht, K. M. Hehir, H. C. M. Nelson, J. M. Sturtevant, R. T. Sauer, *J. Cell. Biochem.* 29, 217 (1985).
4. D. Shortle and B. Lin, *Genetics* 110, 539 (1985); D. Shortle and A. K. Mecker, *Proteins* 1, 81 (1986).
5. A. Pakula, V. Young, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* 83, 8829 (1986).
6. T. Alber, S. Dao-pin, J. A. Nye, D. C. Muchmore, B. W. Matthews, *Biochemistry* 26, 3754 (1987).
7. G. Fermi and M. F. Perutz, *Haemoglobin and Myoglobin* (Clarendon, Oxford, 1981).
8. M. Hollacker and T. E. Creighton, *Biochem. Biophys. Acta* 701, 395 (1982).
9. J. H. Miller, in *The Operon*, J. H. Miller and W. S. Reznikoff, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1978), pp. 31-88.
10.  $\lambda$  repressor consists of an  $\text{NH}_2$ -terminal and  $\text{COOH}$ -terminal domain; each domain contains an independent set of dimerization contacts [C. O. Pabo, R. T. Sauer, J. M. Sturtevant, M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* 76, 1608 (1979); C. O. Pabo, thesis, Harvard University (1980)].
11. C. O. Pabo and M. Lewis, *Nature* 298, 443 (1982).
12. The  $\text{NH}_2$ -terminal domain of  $\lambda$  repressor is minimally comprised of residues 1-92. This is the fragment for which the crystal structure is known. In our studies we use a slightly larger  $\text{NH}_2$ -terminal fragment consisting of repressor residues 1-102. Nuclear magnetic resonance studies show that the 1-92 and 1-102 fragments have the same basic structure and similar dimerization properties (13). The operator binding properties of the 1-92 and 1-102 fragments are also extremely similar (20).
13. M. A. Weiss, C. O. Pabo, M. Karplus, R. T. Sauer, *Biochemistry* 26, 897 (1987); M. A. Weiss, M. Karplus, R. T. Sauer, *ibid.*, p. 890.
14. F. H. Martin, M. M. Castro, F. Aboul-ela, I. Tinoco, *Nucleic Acids Res.* 13, 8927 (1985).
15. In control experiments, we found that cells containing at least 5 to 10 percent of the wild-type activity could survive the phage selection. This figure was determined by Western analysis of lysates of cells containing the wild-type 1-102 gene under control of the inducible *lac* promoter. When expression of 1-102 was induced to a level sufficient to make cells resistant to phage  $\lambda$ KH54, the intracellular level of 1-102 was 5 to 10 percent of that produced from the *lac* promoter.
16. The survival frequency can be somewhat misleading, as some cells containing functional genes do not survive the selection. For example, by screening the unselected candidates in the mutagenesis of positions 86 and 89, we found that approximately 20 percent of the cells contained active protein. By contrast, only 2.4 percent of the cells survived the selection in this experiment.
17. B. Lee and F. M. Richards, *J. Mol. Biol.* 55, 379 (1971); in our studies, accessible surface areas were calculated with the use of the ACCESS program written by T. Richmond.
18. F. S. Gimble and R. T. Sauer, *J. Mol. Biol.*, in press.
19. S. Jordan and C. Pabo, personal communication.
20. R. T. Sauer et al., *Biochemistry* 25, 5992 (1986); C. O. Pabo and E. G. Suchanek, *ibid.* 25, 5987 (1986).
21. M. H. Hecht and R. T. Sauer, *J. Mol. Biol.* 186, 53 (1985).
22. H. C. M. Nelson, M. H. Hecht, R. T. Sauer, *Cold Spring Harbor Symp. Quant. Biol.* 47, 441 (1983).
23. F. M. Richards, *J. Mol. Biol.* 82, 1 (1974); C. Chothia, *Nature* 254, 304 (1975).
24. There are several methods, in addition to our own, that could be used for combinatorial cassette mutagenesis: A. R. Oliphant, A. L. Nussbaum, K. Struhl, *Gene* 44, 177 (1986); K. M. Derbyshire, J. J. Salvo, N. D. F. Grindley, *ibid.* 46, 145 (1986). The use of inosines, in our method, is convenient and efficient, but does lead to some bias in the frequency at which each base is recovered. For example, among the unselected candidates in the mutagenesis of positions 84, 87, and 88, the frequencies at which the four bases were recovered at the mutagenized positions were: 23 percent A, 35 percent C, 20 percent G, and 23 percent T. The methods cited above avoid a pairing bias by performing enzymatic second-strand synthesis.
25. J. W. Ponder and F. M. Richards, *J. Mol. Biol.* 193, 775 (1987).
26. These graphics were produced with the Promodeber molecular graphics program (New England BioGraphics). Coordinates were provided by C. O. Pabo.
27. D. Hanahan, *J. Mol. Biol.* 166, 557 (1983).
28. A. K. Vershon, K. Blackmer, R. T. Sauer, in *Protein Engineering: Applications in Science, Medicine, and Industry*, M. Inouye and R. Sarma, Eds. (Academic Press, Orlando, FL, 1986), pp. 243-256.
29. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463 (1977).
30. D. Eisenberg, R. M. Weiss, T. C. Terwilliger, W. Wilcox, *Faraday Symp. Chem. Soc.* 17, 109 (1982).
31. We thank R. Breyer for providing plasmids and antibodies required for the work, D. Vershon for suggesting the use of inosines for the combinatorial mutagenesis method, and J. Bowie for pointing out the potential uses of the method in structural prediction. We also thank C. Pabo and S. Jordan for providing the coordinates of the  $\text{NH}_2$ -terminal domain and its complex with operator DNA, and thank G. Quigley for help with the accessibility calculations. This work was supported by NIH grant AI-15706 and by a predoctoral grant (to J.R.-O.) from the National Science Foundation.

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*Target Organ Toxicology Series*

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# Free Radical Toxicology

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8

## Free-Radical Defense and Repair Mechanisms

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### INTRODUCTION

Defense and repair systems are critical modulators of cellular oxidative damage. In this chapter, we discuss the functions and interplay of antioxidants and antioxidant enzymes and emphasize the complementary nature of these systems. To explain the need for such diverse antioxidant defense and repair systems, we describe pertinent aspects of the formation and chemistry of biologically relevant oxidants. We also discuss some aspects of free-radical toxicity that is associated with biotransformation of various chemicals. In doing so, we emphasize those oxidants against which cellular antioxidant defense is directed and perhaps account for Nature's selection of specific antioxidant defenses. Detailed discussions of the formation and chemistry of reactive oxidants are presented elsewhere in this volume.

The continual formation of reactive oxygen species is a physiological necessity and an unavoidable consequence of oxygen metabolism. However, when generated in excess, they can be toxic, particularly in the presence of transition metal ions such as iron or copper (elsewhere in this volume); for a review see Halliwell and Gutteridge (1). Since defense systems are present and functioning under normal conditions, endogenous free radicals do not necessarily place biological tissues and cells at risk. However, these defense systems can be overwhelmed during various pathological conditions caused by xenobiotics, anoxia, radiation, and loss of extracellular calcium. Excess generation of free radicals within tissues can cause damage to vital cellular constituents.

It is estimated that nearly 90% of the total  $O_2$  consumed by mammalian species is delivered to mitochondria, where a four-electron reduction to  $H_2O$  by the respiratory chain is coupled to ATP synthesis (2,3). Nearly 4% of mitochondrial  $O_2$  is incompletely reduced by leakage of electrons along the respiratory chain, especially at ubiquinone, forming ROS such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), singlet oxygen, and hydroxyl radical ( $HO^\bullet$ ) (2,3). Richter (4) calculated that during normal metabolism, one rat liver mitochondrion produces  $3 \times 10^7$  superoxide radicals per day. It is estimated that superoxide and hydrogen peroxide steady state concentrations are in the picomolar and nanomolar range, respectively (5). Jones et al. (6) have estimated the hepatocyte steady state  $H_2O_2$  concentration to be up to 25  $\mu M$ . These events are thought to contribute over 85% of the free-radical production in mammalian species. Sohal (7) has concluded that there is a variation in the sites of superoxide or hydrogen peroxide generation among mitochondria from different tissues and species. This is a result of the rate of mitochondrial superoxide production is dependent on at least three variables: (1) ambient oxygen concentration; (2) levels of autoxidizable respiratory carriers, especially ubiquinone; and (3) the redox state of the autoxidizable carriers (2,5,7-9).

Toxic chemicals can cause oxidant formation through several mechanisms. In some instances, metabolism drives the formation of oxygen-containing reactive intermediates through a process known as redox cycling. Many other chemicals can also undergo bioactivation to form biological reactive intermediates that bind to macromolecules and indirectly enhance the formation of oxygen radicals. Some chemicals induce inflammatory responses, in which release of reactive oxygen and nitrogen radicals by stimulated phagocytes constitutes an oxidative challenge. Still other chemicals undergo facile photoexcitation reactions that lead to the formation of either singlet oxygen, free radicals, or both. Oxidant generation through any of these scenarios may lead to the oxidation of critical functional groups on macromolecules, to peroxidation of lipids, and to oxidation of other susceptible cellular constituents.

The evolution of bioactivation processes that form biological reactive intermediates, both free radical and ionic, probably necessitated the concomitant evolution of cellular defense and repair systems for cell survival. All tissues and cells contain defense systems for detoxification of biological reactive intermediates and to prevent or limit cellular damage. Toxic processes have reversible and irreversible features that are a consequence of the interplay with cellular defense and repair systems. Reversible toxicity occurs even with chemicals known as "safe" chemicals. Dose determines whether any chemical causes irreversible toxicity. Irreversible toxicity may cause cell death regardless of what antidotal or preventive measures are taken after exposure of cell or tissues to a sufficient dose of the toxic chemical. Death occurs when loss of cellular integrity occurs to such a degree that free exchange between the intracellular constituents and the surrounding milieu prevents cell survival. We review here the mechanisms by which cells are protected by defense and repair systems to prevent or limit cellular damage and death.

## THE INITIATION AND PROPAGATION OF OXIDATIVE DAMAGE

In this section we describe general characteristics of the origin of free radicals and related oxidants. Rather than provide a comprehensive documentation of the sources of oxidants, we focus on how the common primordial oxidants superoxide and nitric oxide lead to the formation of a number of more reactive oxidants. We also distinguish between the initiation and propagation of oxidative damage and consider the roles of these processes in oxidative injury. This establishes a framework for describing the interlocking, complementary nature of cellular antioxidant defense.

### Superoxide, Nitric Oxide, and Transition Metals as Primordial Oxidant Sources

Major contributors to oxidative injury are (1) xenobiotics or endogenous factors that can cause increased superoxide generation, (2) factors that stimulate the production of nitric oxide, and (3) processes that disrupt heme proteins or other metalloproteins to enhance the contribution of transition metals to oxidant generation. Increased superoxide generation can result from agents that stimulate phagocytic cells including polymorphonuclear leukocytes and macrophages, which generate large quantities of superoxide as a mechanism for destruction of foreign cells. Free-radical generation by neutrophils can be as high as 200 nmol/ $10^6$  cells/h (10,11). It is known that an NADPH oxidase provides the catalysis for the rapid consumption of molecular oxygen (12,13). Even nonphagocytic cells can be stimulated by agents such as phorbol esters to increase superoxide production through the conversion of xanthine dehydrogenase to xanthine oxidase (14). Chemicals also can induce superoxide formation by catalyzing electron transfer from cellular redox proteins to molecular oxygen (redox cycling), as discussed elsewhere in this volume.

Many of the same stimuli that induce macrophages to produce superoxide also stimulate the production of nitric oxide by inducible nitric oxide synthetase (see Morris and Billiar, 15, for a recent review). Nitric oxide produced in relatively high yield by these inducible enzymes may react with oxygen or superoxide to yield more reactive oxidants, as described later. Finally, oxidants, nonoxidizing reactive intermediates, and other mediators of cellular injury may disrupt hemoproteins and other metalloproteins to release transition metal ions, particularly iron and copper, which may amplify damage by catalyzing the formation of highly reactive radicals. Reactions of superoxide, nitric oxide, and metals lead to oxidative injury by forming secondary oxidants that are believed to be responsible for actually causing most biological oxidative damage. These reactions and some of their intermediates are key control points in the initiation and propagation of oxidative damage. These critical reactions and intermediates also are often the targets of antioxidant defense.



### Reactions of Superoxide, Nitric Oxide, and Metals: Formation of Secondary Oxidants

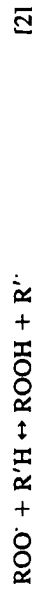
Superoxide is itself a reasonably strong oxidant ( $E^\circ = 940 \text{ mV}$  at pH 7) (16), but most of its pro-oxidant chemistry is thought to be due to formation of its conjugate acid  $\text{HOO}^\cdot$  ( $\text{p}K_a$  4.9), which may initiate lipid peroxidation by hydrogen abstraction from hydroperoxides. Superoxide decomposition gives rise to even more reactive oxidants. Enzyme-catalyzed and nonenzymatic dismutation yield hydrogen peroxide, a nonradical pro-oxidant that may freely diffuse across membranes. Metal-catalyzed cleavage of hydrogen peroxide (the Fenton reaction) forms hydroxyl radical or similarly reactive high-valent metal-oxo complexes, which are the most reactive oxidants known in biological systems (Koppenol, chapter 1, this volume).

Superoxide also may react at diffusion limited rates with nitric oxide to form the highly reactive nonradical peroxynitrite (17). Peroxynitrite is an excellent oxidant for biological thiols, and its conjugate acid peroxynitrous acid forms a reactive oxidant with reactivity similar to hydroxyl radical (18). In addition to its reaction with superoxide, nitric oxide also may autoxidize to nitrogen dioxide, which is a highly reactive initiator of free-radical reactions (19).

Transition metal ions may greatly enhance oxidative damage in two general ways. First, they may reductively cleave hydrogen peroxide and alkyl hydroperoxides to hydroxyl and alkoxy radicals, respectively (i.e., the Fenton reaction, reviewed by Koppenol, chapter 1, this volume). The extent to which this reaction occurs in vivo depends on the availability of metal ions. However, in all but the most stringently demetalized experimental systems, metal-catalyzed cleavage of lipid hydroperoxides probably is the driving force for most lipid peroxidation studied in vitro (20–22). Metal-catalyzed cleavage of lipid hydroperoxides greatly amplifies lipid peroxidation, and analogous reactions may contribute significantly to the autoxidation of proteins and DNA as well. Metals also may directly oxidize thiols to thyl radicals, which may add oxygen to form more highly oxidizing intermediates (reviewed in refs. 16 and 23). The reduced forms of the metal ions formed by these reactions may participate in Fenton chemistry as described earlier.

### Formation and Reactions of Peroxyl Radicals: Propagation of Oxidative Damage

The reactive intermediates discussed earlier all contribute to oxidative damage either by direct reaction with oxidizable biomolecules (hydroxyl radical, alkoxy radical, peroxynitrite, hydroperoxyl radical, nitrogen dioxide) or by serving as immediate precursors to radicals (hydrogen peroxide, organic hydroperoxides, peroxynitrous acid). The radical species react with lipids, proteins, DNA or other biomolecules either by addition, hydrogen abstraction, or electron transfer to form (primarily) biomolecule-derived, carbon-centered radicals. In aerobic environments, these carbon centered radicals reversibly add oxygen to form peroxyl radicals, which then react with adjacent biomolecules:



This process is termed *propagation* and is normally considered in terms of lipid peroxidation (see Sevanian and McLeod, chapter 4, this volume). However, the same sequence of reactions also can contribute to the spread of oxidative damage in proteins, DNA, and other biomolecules. A free-radical chain initiated by a single hydroxyl radical in a lipid membrane thus may lead to over 20 propagation cycles before the chain is terminated (24). All of the other radicals discussed earlier can initiate radical chains, which then are propagated in the same manner. It is not surprising in this context that peroxyl radical propagation reactions can contribute the bulk of oxidative damage regardless of the specific initiating oxidants involved.

### THE ORGANIZATION OF ANTIOXIDANT DEFENSE

Cellular antioxidant defenses are organized into several tiers against oxidant challenges. The juxtaposition of oxidant challenge and antioxidant defense is illustrated in Figure 1. For clarity, we have greatly simplified the highly complex chemistry involved in the initiation and propagation of oxidative damage. Key elements of oxidative challenges are described earlier and depicted in Figure 1. These include (1) the formation of primordial radicals superoxide and nitric oxide, (2) the generation of highly reactive secondary intermediates (e.g., hydroxyl and hydroperoxyl radical, peroxynitrite), which may initiate free-radical chain reactions, and (3) amplification by peroxyl radical dependent chain propagation. Another key element of oxidant challenge is the role of transition metals both in forming highly reactive secondary intermediates (e.g., Fenton chemistry) and in amplifying the propagation of oxidative damage.

Antioxidant defenses are directed against several aspects of the oxidant challenge. A general observation is that the enzyme-mediated antioxidant defenses are directed against the primordial initiator superoxide and the less reactive secondary mediators hydrogen peroxide and organic hydroperoxides. Small-molecule chain-breaking antioxidants are instead directed primarily against peroxyl radicals involved in radical propagation. Cellular antioxidant defenses thus serve complementary functions within the context of a multitiered oxidant challenge. Characteristics of the individual components of cellular antioxidant defense are described later. More extensive discussion of the antioxidant roles of glutathione, vitamin E, and ascorbate is provided in succeeding sections.

### Superoxide Dismutases, Catalase and Glutathione Peroxidases

Superoxide dismutases (SOD) catalyze the dismutation of superoxide to oxygen and hydrogen peroxide. A major protective benefit is derived from enzymatic

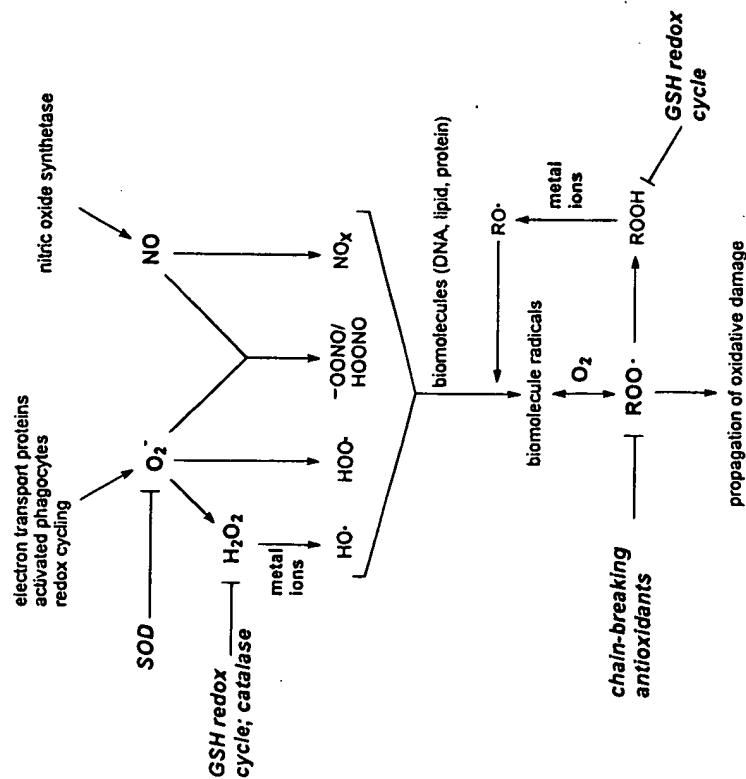


FIG. 1. Sites of blocking oxidant challenges by antioxidant defenses.

catalysis, since the nonenzymatic rate is approximately four orders of magnitude smaller at pH 7.4 (25). Mammalian SOD enzymes include a homodimeric Cu,ZnSOD in the cytosol and a homotetrameric MnSOD in mitochondria (26). Extracellular fluids contain a tetrameric, glycosylated form of the Cu,Zn enzyme (27). The mitochondrial MnSOD is highly inducible by cytokines such as tumor necrosis factor (28) and by mediators of acute oxidative stress, such as superoxide or hydrogen peroxide (29). Catalase is a hemoprotein found in peroxisomes of eukaryotic cells and catalyzes the conversion of hydrogen peroxide to water and oxygen. This enzyme also can be induced in response to cellular hydrogen peroxide exposure (29).

Glutathione peroxidases are selenoproteins found in essentially all tissues. Four isozymes of with glutathione peroxidase activity have been characterized: (a) the classical cellular glutathione peroxidase, GSHPx-1, (b) the phospholipid hydroperoxide glutathione peroxidase, GSHPx, (c) the plasma glutathione peroxidase, GSHPx-P, and (d) GSHPx-GI (30).

The best known of these, glutathione peroxidase, is a homotetramer consisting of 22-kD subunits, each with one selenocysteine residue (31). The enzyme is found

in cytosol and mitochondria and reduces hydrogen peroxide and some organic hydroperoxides to water and alcohols, respectively. A similar enzyme found in extracellular fluids and in plasma shares sequence homology with the intracellular enzyme, but is a separate gene product (32). Another enzyme, phospholipid hydroperoxide glutathione peroxidase, is a monomeric protein containing one selenocysteine (33). This enzyme reduces phospholipid hydroperoxides to the corresponding alcohols.

Ursini et al. (33) has purified and characterized an interfacial glutathione peroxidase. This enzyme has been shown to reduce linoleic acid hydroperoxides, cumene hydroperoxide, *tert*-butyl hydroperoxide, and hydrogen peroxide. However, this enzyme, which does not conjugate CDNB with GSH (34), displays glutathione peroxidase activity toward cumene hydroperoxide, hydrogen peroxide, and lipid hydroperoxides and is distinct from the classical glutathione peroxidase (35). Evidence suggests that the enzyme is interfacial in character and can interact directly with liposomes to reduce phospholipid hydroperoxides (33). The addition of this protein to microsomal incubation mixtures inhibited lipid peroxidation (34). Substrate specificities indicate that this enzyme is distinct from the nuclear glutathione transferase (36). In addition to the selenium-dependent glutathione peroxidases, a related extracellular selenoprotein, selenoprotein-P, is found in plasma and in extracellular fluids (37). This monomeric 41 kD glycoprotein contains 10 selenocysteines, and enzyme levels are highly sensitive to changes in dietary selenium status. Although a specific antioxidant activity has not been established for this protein, it is postulated to exert antioxidant effects (37). The soluble intracellular form of Selenoprotein peroxidase is generally regarded as an indispensable defense against hydrogen peroxide (31). Reduction of phospholipid hydroperoxides by phospholipid hydroperoxide glutathione peroxidase, together with peroxyl radical scavenging by vitamin E (discussed later), is thought to constitute a highly efficient defense against membrane lipid peroxidation (38). Weitzel and Wendel (39) have reported findings that phospholipid hydroperoxide-glutathione peroxidase activity regulates the activity of 5-lipoxygenase via regulating the tone of endogenous hydroperoxides. Nevertheless, the relative importance of these different selenoproteins in antioxidant protection remains poorly understood. Awad et al. (40) recently reported that severe dietary selenium deficiency reduced glutathione peroxidase activity to less than 2% of control values without increasing hepatic lipid peroxidation, whereas vitamin E deficiency did increase lipid peroxidation. In another experiment, diquat toxicity in chronically selenium deficient rats was reduced by pretreatment with selenium 12 h prior to diquat exposure. However, the selenium pretreatments did not affect either selenium glutathione peroxidase or phospholipid hydroperoxide glutathione peroxidase, but instead increased plasma levels of selenoprotein-P. A better understanding of the antioxidant role of selenium awaits a clarification of the true function of selenoprotein-P and extracellular selenium glutathione peroxidase and of their interplay with intracellular glutathione peroxidases.

Michiels et al. (41) have reviewed the importance of the defense enzymes Selenoprotein peroxidase, catalase, and Cu,Zn-SOD for cell survival against oxidative

stress. From the evidence, they suggest that each enzyme has a specific as well as an irreplaceable function. In part, roles of the enzymes were assessed with the aid of specific enzyme inhibitors including aminotriazole (42) for catalase, diethyldithiocarbamate (43) for Cu,Zn-SOD, mercaptosuccinate (44) for glutathione peroxidase, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (45) for glutathione reductase, and buthionine sulfoximine (BSO) (46) for GSH synthesis. In transfection studies in which overexpression of a specific enzyme is studied, the physiological responses indicate that the metabolism of reactive oxygen species may have a critical balance. For example, Mn-SOD-transfected mouse cells overexpressing SOD activity were found to be more resistant to hyperoxia (47) and paraquat (47,48). However, Cu,Zn-SOD-enriched bacteria displayed increased sensitivity to hyperoxia (49) and paraquat (50). Warner (51) concurs that SOD has an important role in defense against degenerative disease, but much remains to be understood concerning effects of manipulation of SOD expression as an intervention. Transfection experiments could therefore cause increased or decreased toxicity depending on relative concentrations of hydrogen peroxide and superoxide. In addition, iron (or copper) has the potential for a major influence by being optimized in its redox ratio ( $\text{Fe}^{2+}/\text{Fe}^{3+}$ ) and involving various constituents for increasing the reactivity of hydrogen peroxide. Luo et al. (52) have proposed that hydrogen peroxide toxicity is associated with three chemically distinct types of oxidants formed by iron-mediated Fenton reactions in the presence of DNA.

### Small-Molecule Antioxidants

Numerous small molecules (<1000 MW) with high reactivity toward oxidants have been described. Three of these, vitamin E, ascorbic acid, and glutathione, play essential antioxidant roles in tissues and in extracellular fluids. The functions of these are discussed in detail next. Numerous other molecules, including carotenoids (53,54), dihydrolipoic acid (55), flavonoids (56), plant polyphenols (57), and ubiquinol (58,59), exert antioxidant effects in vitro and may assume important antioxidant roles in vivo under conditions of increased dietary intake or pharmacological supplementation. Some products of intermediary metabolism, such as bilirubin (60) and uric acid (61), that are toxic at high levels may nevertheless exert antioxidant effects in some tissue microenvironments. Many drugs and other chemicals have been shown to have antioxidant properties in vitro and may also contribute to antioxidant defense under certain circumstances.

In contrast to antioxidant enzymes, which scavenge superoxide and hydrogen peroxide, small molecule antioxidants act for the most part as scavengers of secondary oxidants and inhibitors of radical chain propagation. Such molecules may be classified as *chain-breaking antioxidants*, which are defined as compounds that act by trapping peroxyl radicals. As discussed earlier, peroxyl radicals are the principal chain-propagating species encountered in oxidant challenges and probably the most common ultimate mediators of oxidative damage. Chain breaking antioxidants typi-

cally function in a two-step sequence in which the antioxidant traps one peroxyl radical to form an antioxidant-derived radical [Eq. (3)], which then traps a second radical to form nonradical products [Eq. (4)].



Each reaction terminates a radical chain and thus may prevent as many as 20 or more subsequent oxidations. Chain-breaking antioxidants can be effective at relatively low concentrations. For example, membranes typically contain about 1  $\alpha$ -tocopherol per 1000 phospholipids (62,63).

Because small-molecule antioxidants are known to react at high rates with many oxidants (hydroxyl radicals, peroxynitrite, alkoxyl radicals, peroxyl radicals), it perhaps seems surprising that these compounds should act primarily as chain-breaking antioxidants. The apparent specificity of most small-molecule antioxidants for reaction with peroxyl radicals as opposed to other radicals (e.g., hydroxyl radical) is due largely to kinetics. Peroxyl radicals react at relatively slow rates with many biomolecules (e.g.,  $k \sim 10^2 \text{ M}^{-1} \text{ s}^{-1}$  for linoleic acid (64), but at much higher rates with antioxidants (e.g.,  $k \sim 10^5\text{--}10^6 \text{ M}^{-1} \text{ s}^{-1}$  for  $\alpha$ -tocopherol, depending on the reaction medium (65). Reaction of peroxyl radicals with the antioxidant is kinetically favored, even though the concentration of oxidizable lipid greatly exceeds that of the antioxidant. In contrast, hydroxyl radicals react with most biomolecules and antioxidants at near the diffusion controlled rate [ $k \sim 10^9 \text{ M}^{-1} \text{ s}^{-1}$  (66,67)], so there is no kinetic preference for reaction with the antioxidant. Indeed, since other biomolecules outnumber antioxidant molecules, quenching of hydroxyl radicals would occur infrequently. The antioxidant instead would be much more likely to trap peroxyl radicals formed subsequent to initial hydroxyl radical attack. In view of these considerations, the frequently used term "hydroxyl radical scavenger" is probably a misnomer, at least in reference to actions in vivo. Only when relatively high concentrations of scavenger are employed in vitro would effective scavenging of hydroxyl radicals occur.

Small molecule antioxidants react with radicals by a variety of mechanisms [Eqs. (5–10)]. Phenolic antioxidants such as  $\alpha$ -tocopherol and thiols such as glutathione usually quench radicals by hydrogen atom transfer (although the reaction also may occur by rapid electron transfer followed by proton transfer) [Eqs. (5) and (6)] (23,68).



The water soluble antioxidants ascorbate and urate instead react by electron transfer [Eqs. (7) and (8)] (16,61).



Carotenoids react by a combination of electron transfer and radical addition to the carotenoid polyene system [Eqs. (9) and (10)] (69,70).

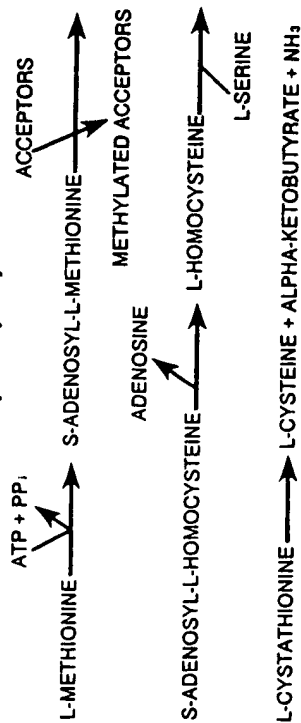


The radical intermediates generated by all these reactions may react with additional radicals in radical-radical termination reactions (e.g., reaction 4) or may undergo disproportionation or reductive "repair" reactions. These reactions of  $\alpha$ -tocopherol, glutathione, and ascorbate and their radical intermediates are considered in detail in the following sections.

### CELLULAR GLUTATHIONE AND OTHER THIOLS AS DEFENSE AND REPAIR AGENTS

Depending on the cell type, the intracellular concentration of glutathione is maintained in the range of 0.5–10 mM (71). Concentrations in the liver are 4–8 mM. Because of the redox status of glutathione that is maintained by intracellular glutathione reductase and NADPH, nearly all the glutathione is present as reduced glutathione (GSH) with less than 5% of the total is present as glutathione disulfide (GSSG). Constant production of GSSG is a result of continual endogenous production of superoxide from oxygen leading to the formation of hydrogen peroxide and lipid hydroperoxides. The GSH content of various organs and tissues represents at least 90% of the total nonprotein, low-molecular-weight thiols. The GSH content of liver is nearly twice that found in kidney and testes and over threefold greater than in the lung. The importance of hepatic GSH for protection against free radicals has been reviewed extensively (72–75).

The cystathionine pathway is of major importance to pathways of free-radical generation that can cause loss of GSH. Depletion of GSH by rapid conjugation can increase synthesis of GSH to rates as high as 2–3  $\mu\text{mol/h/g}$  wet liver tissue (76). The cysteine pool in the liver, which is about 0.2  $\mu\text{mol/g}$ , has an estimated half-life of 2–3 min at such high rates of synthesis of GSH (72). Although the cystathionine pathway appears to be highly responsive to the need for cysteine biosynthesis in the liver, the organ distribution of the pathway may be limited.



In mammals, such as rats, the liver is the main site of cysteine biosynthesis, which occurs via the cystathionine pathway as shown in the preceding scheme. Maintenance of high concentrations of GSH in the liver, in association with high rates of GSH secretion into plasma and extensive extracellular degradation of GSH and GSSG, supports the concept that liver GSH is a physiological reservoir of cysteine. This idea, which was proposed originally by Tateishi et al. (77) and Higashi et al. (78), was that cells possess two pools of GSH. One has a fast (2-h) and the other a slow (30-h) turnover (78,79). Meredith and Reed (80) observed that in freshly isolated rat hepatocytes the mitochondrial pool of GSH (about 10% of the total cellular pool) had a half-life of 30 h while the half-life of the cytoplasmic pool was 2 h. They concluded that the mitochondrial pool might represent the stable pool of GSH observed in whole animals. Further, cysteine has a sparing effect on the requirement of the essential amino acid methionine in the rat (81). This observation is in agreement with the unidirectional process of trans-sulfuration in which methionine sulfur and serine carbon are utilized in cysteine biosynthesis via the cystathionine pathway. For reviews see Reed and Beatty (72) and Reed (75).

In vivo treatment of rats with an inhibitor of  $\gamma$ -glutamyl transpeptidase (AT-125) prevents degradation of GSH in plasma leading to massive urinary excretion of GSH (82a). This treatment also lowers the hepatic content of GSH because it inhibits recycling of cysteine to the liver (80). A physiologic decrease in interorgan recycling of cysteine to the liver for synthesis of GSH also may account in part for the decrease of hepatic GSH during starvation and for the marked diurnal variation in concentration of GSH in liver. The nadir occurs in the late afternoon, whereas the early morning peak occurs shortly after the animals are fed. The efflux of liver GSH and metabolism of the resulting plasma GSH and GSSG appears to help insure a continuous supply of plasma cysteine. This cysteine pool should in turn minimize the degree of fluctuation of GSH concentrations within the various body organs and cell types that require only cysteine or cystine, or both, rather than methionine for synthesis of GSH.

Glutathione deficiency can be achieved in vivo by the administration of BSO. When newborn rats or guinea pigs are treated with BSO a GSH deficiency develops and the animals develop multiorgan failure and die within a few days. Death can be prevented by the administration of ascorbate (82b).

A controversial approach to assessing the potential for chemicals to cause free-radical damage in vivo is to chemically intoxicate an intact animal and then to measure products of lipid peroxidation in microsomes prepared from the intoxicated animal. In this manner, the depletion of glutathione in vivo with agents that form glutathione conjugates enhances subsequent lipid peroxidation in vitro. Results from such experiments show consistently that an in vivo threshold of 1  $\mu\text{mol GSH/g}$  liver is associated with spontaneous lipid peroxidation in microsomes (83). This critical value of GSH is about 20% of the initial concentration of GSH. Addition of exogenous GSH inhibited the lipid peroxidation in vitro in a concentration-dependent manner; 1 mM GSH yielded 50% inhibition. There also is observed a strong enhancement of spontaneous lipid peroxidation in phenobarbital-induced rats.

The role of GSH as a defense against oxidative stress generated by free radicals has been quantified by the measurement of lipofuscin. A hypothesis for lipofuscinogenesis that was postulated by Brunk et al. (84) involves the interplay of two processes: (1) the intracellular production of superoxide and hydrogen peroxide and (2) secondary lysosomes that degrade lipids and proteins to a poorly defined substance known as lipofuscin (85). The loss of GSH by BSO treatment of cardiac myocytes resulted in an increase in lipofuscin that appears associated with loss of GSH-dependent defense against increased levels of hydrogen peroxide (85).

### GLUTATHIONE REDOX CYCLE DEFENSE AGAINST FREE-RADICAL-INDUCED EVENTS

A major defense system against endogenous reactive oxygen species is the glutathione redox cycle (Figure 2) (for a review see Reed, 86). Support for the role of the glutathione redox cycle in defense is that GSH depletion to about 20–30% of normal level of glutathione can impair the cell's defense against the toxic actions of both biological reactive intermediates and reactive oxygen species and may lead to cell injury and death. Endogenous free-radical production, which is a normal physiological process, is a consequence of aerobic metabolism that occurs mostly in the mitochondria of eukaryote cells. Mitochondrially generated  $H_2O_2$ , if not decomposed, can lead to the formation of radicals that cause damage to membranes, nucleic acids, and proteins, and alter their functions. A major protective role against exogenous free radicals, which are generated by bio-reduction of many xenobiotics followed by redox cycling, is also provided by the ubiquitous glutathione redox cycle (Figure 2). This cycle utilizes NADPH- and, indirectly, NADH-reducing equivalents in the mitochondrial matrix as well as the cytoplasm to provide GSH by the glutathione reductase-catalyzed reduction of GSSG. When the glutathione redox cycle is functioning at maximum capacity to eliminate hydrogen peroxide, a major regulatory effect is imposed on other NADPH-dependent pathways.

Glutathione reductase, which is important in the defense against oxygen-derived free radicals by GSH, is itself regulated by the redox status of the cell. Being similar to other reductases such as nitrate, nitrite, and NADP<sup>+</sup> reductase, GSH reductase is inactivated upon reduction by its own electron donor, NADPH. It has been proposed that this autoinactivation of glutathione reductase by NADPH and the protection as well as reactivation by GSSG regulates the enzyme *in vivo* (87). The activity of glutathione reductase may reflect the physiological needs of the cell especially during oxidative stress. For example, 40–50  $\mu M$  intracellular NADPH inactivates glutathione reductase in the absence of GSSG and decreases glucose metabolism via the hexose monophosphate pathway. The physiological ratio of GSSG:GSH should provide sufficient GSSG at this level of NADPH to permit retention of significant glutathione reductase activity by preventing inactivation (87).

Cytosolic glutathione peroxidase is a selenium-dependent enzyme that is extremely specific for glutathione and is capable of rapidly detoxifying hydrogen

### CELLULAR PROTECTIVE SYSTEMS

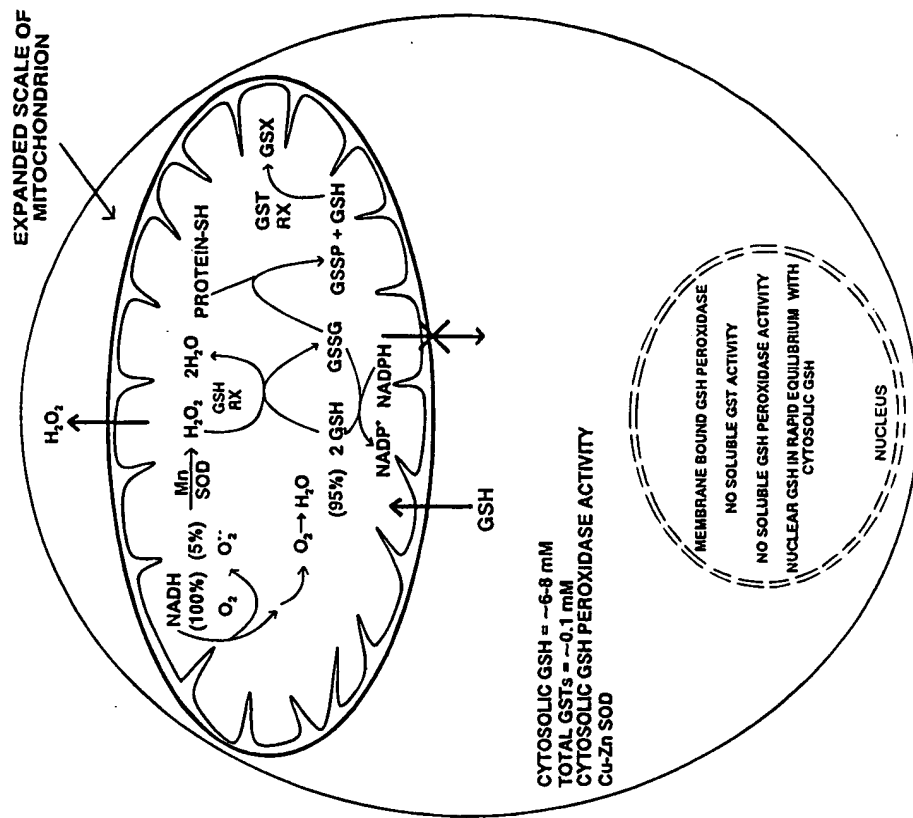


FIG. 2. Cellular protective systems and the glutathione redox cycle. MnSOD in the mitochondria and Cu-ZnSOD in the cytosol provide enzymatic conversion of superoxide to hydrogen peroxide, which is detoxified by the glutathione redox cycle components glutathione reductase and peroxidase present in the mitochondria matrix (shown) and the cytosol (not shown).

peroxide and certain hydroperoxides as a partner in the glutathione redox cycle with glutathione reductase. As mentioned earlier, selenium-dependent glutathione peroxidase activity is the result of the expression of multiple isozymes.

The antitumor benzantraquinone, adriamycin, undergoes rapid bio-reduction by NADPH-dependent cytochrome P-450 reductase with concomitant consumption of

oxygen (88). Adriamycin cytotoxicity may be the result of free radicals formed by bioreduction that overwhelm the cellular antioxidant capacity, including that portion provided by the glutathione redox cycle. Inactivation of glutathione reductase with BCNU has permitted the demonstration of the protective role of the glutathione redox cycle against an adriamycin-mediated challenge (89,90). Depletion of GSH concurrently with inactivation of glutathione reductase can enhance the cellular injury mediated by adriamycin-generated radicals in isolated hepatocytes (90).

The intracellular concentration of GSH in isolated hepatocytes has been examined under conditions that result in enhanced free-radical production. Production of malondialdehyde, which is an index of lipid peroxidation, can be stimulated by addition of a glutathione depletor, diethyl maleate (89). This observation suggests that intracellular concentrations of GSH under these conditions are important for membrane and cellular integrity. That is, GSH protects against free-radical damage to unsaturated fatty acid moieties in biological membranes.

#### Defense by Glutathione S-Transferases

GSH-dependent protection against lipid peroxidation has been demonstrated in mitochondria (91-93), nuclei (94), microsomes (91,93-98), and cytosol of rat liver (99-101). Lipid peroxidation induced in mitochondria also is inhibited by respiratory substrates such as succinate, which leads indirectly to reduction of ubiquinone to ubiquinol. The latter is a potent antioxidant (102-104). The essential factor in preventing accumulation of lipid peroxides and lysis of membranes in mitochondria, however, is glutathione peroxidase (105). Although the prevention of free-radical attack on membrane lipids may occur by an electron shuttle that utilizes vitamin E and GSH in microsomes, similar activity may not be capable of inhibiting peroxidation in mitochondria (96,106). Instead, mitochondrial GSH S-transferase(s) may prevent lipid peroxidation in mitochondria by a non-selenium glutathione-dependent peroxidase activity. Three GSH S-transferases have been isolated from the mitochondrial matrix (107), and nearly 5% of the mitochondrial outer membrane protein consists of microsomal glutathione S-transferase (108). GSH S-transferase in the outer mitochondrial membrane could provide the GSH-dependent protection of mitochondria by scavenging lipid radicals by a mechanism that requires vitamin E and is abolished by bromosulphthalein (108).

A limited number of studies have focused on the susceptibility of the cell nucleus to lipid peroxidation. The nuclear membrane regulates the transport of mRNA into the cytoplasm and aids in the process of nuclear division. DNA is also frequently associated with certain regions of the nuclear membrane (109), and it seems likely that nuclear membrane peroxidation may disrupt many of these critical functions. The proximity of the nuclear membrane to DNA could also contribute to the interaction of DNA with reactive compounds generated in lipid peroxidation. Several studies indicate that such lipid peroxidation products can alter the structure and function of DNA (110-113). This fact is of importance since hydroxyl radicals diffuse an average of only 60 Å before reacting with cellular components (114). Assays for

8-hydroxy-2'-deoxyguanosine as a biomarker of oxidative DNA damage include *in vivo* studies with urine samples (115). Nuclear peroxidation may also increase interactions between more stable peroxidation products and DNA. The cytosolic enzymes aldehyde dehydrogenase (116), glutathione transferase (117), and glutathione peroxidase (91) have all been shown to metabolize various reactive lipid peroxidation products. Such cytosolic enzymes may metabolize peroxidation products generated throughout the cell before they diffuse into the nucleus and interact with DNA.

Glutathione protection of isolated rat liver nuclei against lipid peroxidation is abolished by exposing isolated nuclei to the glutathione transferase inhibitor *S*-octylglutathione (36). *S*-Octylglutathione also inhibited nuclear glutathione transferase activity and glutathione peroxidase activity. A large percentage of the glutathione transferase activity associated with isolated nuclei was solubilized with 0.3% Triton X-100. Since this treatment removes nuclear membranes while preserving the integrity of the remaining nucleus, it appears that the peroxidase activity is associated with the nuclear membrane. This activity in conjunction with GSH may contribute to the inhibition of lipid peroxidation in nuclear membranes and thereby preserve the integrity of this important membrane system. Increasing evidence suggests that this inhibition of peroxidation may in turn protect the structure and function of DNA.

Endogenous  $\alpha$ -tocopherol levels in isolated rat liver nuclei have been measured and found to be 0.045 mol E (mol  $\alpha$ -tocopherol per mol phospholipid  $\times$  100) (36). This value corresponds to 970 polyunsaturated fatty acid (PUFA) moieties to one molecule of  $\alpha$ -tocopherol in the nuclear membrane. These values are higher than values reported for rat liver microsomes (3313) (63) and mitochondria (2100) (118). A threshold level of 0.085 mol% for the prevention of NADPH-induced lipid peroxidation was established for isolated nuclei. That value could be lowered to 0.040 mol% when 1 mM GSH was added to assist in the inhibition of lipid peroxidation. The ability of GSH to enhance  $\alpha$ -tocopherol-dependent protection against nuclear lipid peroxidation appears to be mainly by a "sparing" effect on the near threshold level of  $\alpha$ -tocopherol in the nuclear membrane.

Since lipid hydroperoxides can initiate lipid peroxidation, the reduction of these compounds can contribute to the inhibition of peroxidation (Figure 1). If glutathione peroxidase activity is associated with the phospholipid bilayer of the nuclear membrane, such an association may contribute to the ability of the peroxidase to reduce lipid hydroperoxides. Thus, the association of a glutathione-dependent peroxidase with membranes may encourage the reduction of lipid hydroperoxides located within lipid bilayers.

#### Glutathione Compartmentation and Defense Against Free-Radical-Induced Injury: Mitochondrial GSH

Several studies have shown that mitochondrial GSH functions as a discrete pool separate from cytosolic GSH. A report by Jocelyn (119) demonstrated that

mitochondrial GSH is impermeable to the inner membrane following isolation of mitochondria. However, Kurosawa et al. (120) have reported the transport of glutathione across mitochondrial membranes. Garcia-Ruiz et al. (121) have evidence that the rat hepatic mitochondrial carrier for reduced glutathione (GSH) transport is distinct from the sinusoidal and canalicular transporters. Wahlander et al. (122) reported the concentration of mitochondrial GSH (10 mM) is higher than cytosolic GSH (7 mM). As previously mentioned, studies by Meredith and Reed (80) demonstrated different rates of GSH turnover in the cytosol and mitochondria confirming the existence of separate intracellular GSH pools. The ratio of GSH:GSSG in mitochondria is approximately 10:1 under normal (untreated) conditions. As reported by Olafsdottir et al. (123), unlike cytosolic GSSG, GSSG is not effluxed from the mitochondrial matrix compartment. This study demonstrated that during oxidative stress induced with *t*-butylhydroperoxide, GSSG is accumulated in the mitochondrial matrix and eventually reduced back to GSH. However, as the redox state of the mitochondria increases, an increase in protein mixed disulfides is also observed. This study concluded that mitochondria are more sensitive to redox changes in GSH:GSSG than the cytosol and therefore mitochondria may be more susceptible to the damaging effects of oxidative stress. These findings suggest that under certain experimental conditions, irreversible cell injury due to oxidative challenge may result from irreversible changes in mitochondrial function.

Mitochondria play a critical role in cellular defense free radicals and associated nonradical oxidants. One of the difficulties in experimentally demonstrating the essential role of mitochondrial GSH has been the inability to selectively deplete the mitochondrial pool of GSH. Recently, Shan et al. (124) have utilized the mitochondrial 3-hydroxybutanoate dehydrogenase to generate a GSH-depleting agent, 3-oxo-4-pentenolate, from (*R,S*)-3-hydroxy-4-pentenolate. By a Michael acceptor reaction of 3-oxo-4-pentenolate with GSH, the mitochondrial GSH pool is selectively depleted. Because mitochondria are a major site for the generation of reactive oxygen species they are susceptible to injury from free radicals. Because of the lack catalase in mitochondria, the entire burden for defense is dependent upon the glutathione redox cycle. Shan et al. (124) observed that the depletion of the mitochondrial, but not the cytosolic, glutathione pool potentiated the cytotoxicity of *tert*-butyl hydroperoxide. As first proposed by Meredith and Reed (80,90), oxidant and electrophile cytotoxicity was correlated with the depletion of GSH in mitochondria but not the cytosol. For reviews see Reed (73-75). In agreement with the mitochondria being a target for free-radical injury, transgenic mice that express high levels of Mn-SOD activity in mitochondria are protected from adriamycin-induced cardiac toxicity (125).

Since mitochondria contain the enzymes and cofactors necessary for the GSH/GSSG redox cycle (126) but do not contain catalase (127), we may assume that a primary function of mitochondrial glutathione (GSH) is the detoxification of endogenously produced  $H_2O_2$ . This redox cycle also protects protein sulfhydryls from oxidation (128).

The mitochondrial glutathione redox cycle has a role in regulating mitochondrial

oxidations. Various oxidants decrease  $O_2$  uptake by isolated mitochondria and cause a complete turnover of GSH via glutathione peroxidase every 10 min (129). It appears that a continuous flow of reducing equivalents through the glutathione redox cycle is balanced by a continuous formation of mitochondrial NADPH, which is needed for glutathione reductase activity. In addition, metabolism of hydrogen peroxide in mitochondria poses a regulatory function in regard to the oxidation of substrates by lipamide-dependent ketoacid oxidases (129), which generate NADPH-reducing equivalents. The entire NADPH:NADP<sup>+</sup> pool may turn over at least once every minute during a maximum oxidant challenge.

The extent to which bioreduction utilizes mitochondrial reducing equivalents is still uncertain, but evidence is increasing that such effects are very important and relate to both calcium and protein thiol homeostasis of mitochondria. For example, the loss of NADPH, which occurs following addition of menadione to isolated mitochondria, is not related to its consumption via the glutathione redox cycle but more likely is related to bioreductive metabolism by NADH-ubiquinone oxidoreductase (130,131).

### Protein Thiols and Toxicity

Thiol groups are well known to be important for normal protein functions, and increasing evidence supports the vital importance of these thiols for cell viability during cytotoxic events. Protein inactivation by oxidation of protein cysteinyl thiols has been shown to occur in more than 240 enzymes (132,133). Membrane-bound enzymes are damaged during lipid peroxidation, and evidence of vitamin E protection strongly supports a free-radical mechanism for protein damage via oxidative stress (134). Oxidative stress can cause loss of protein functions by damaging amino acid residues other than cysteine including methionine, tryptophan, and histidine. An important aspect of such damage is that lipid peroxidative events can amplify free-radical processes that propagate chain reactions. Failure to terminate free-radical processes with a chain-breaking antioxidant, such as vitamin E, can lead to 4 to 10 propagation events occurring per initiation and thus each initiation is amplified (92). Since the reduction of lipid hydroperoxides by GSH utilizes NADPH for the regeneration of GSH from GSSG, the rate of NADPH production can be limiting during oxidative stress (86). Therefore GSSG may be transported from the cell, especially the liver parenchymal cell, when not reduced due to limited levels of NADPH (135). Decreased availability of NADPH and GSH can impair other GSH-dependent detoxication pathways including metabolism of hydrogen peroxide (6), decreased protection of thiols in protein (136,137), and decreased reaction with free radicals (97). Thus, energy-dependent processes involving NADPH, GSH, and thiols in proteins appear to be critically involved in cellular homeostasis during chemical-induced toxicity.



### ANTIOXIDANT PROTECTION BY VITAMINS E AND C

Vitamin E is the name given to a family of natural products comprising the tocopherols and tocotrienols. The most potent of these in animal bioassays is  $\alpha$ -tocopherol, which has the highest rate constant for reaction with peroxyl radicals (138). Tocopherols are not synthesized *de novo* in animals, and tissue levels generally reflect dietary intake of grains and plant-derived oils, which are the best natural sources. The liver plays a central role in  $\alpha$ -tocopherol distribution by incorporating dietary  $\alpha$ -tocopherol into lipoproteins, which then deliver  $\alpha$ -tocopherol to tissues. Other tocopherols, principally  $\gamma$ -tocopherol, may be delivered directly to tissues via chylomicrons.

Dietary  $\alpha$ -tocopherol deficiency enhances the susceptibility of biological membranes to oxidative damage *in vitro* and *in vivo* (for a review, see Chow, 139). Diplock (140), and Rice-Evans and Diplock (141) have reviewed the status of antioxidant nutrients and disease prevention. This section focuses on the interaction of antioxidants for protection against free-radical damage.

#### $\alpha$ -Tocopherol Threshold and Antioxidant Effect

Although regulation of  $\alpha$ -tocopherol distribution at the cellular level is poorly understood, membrane  $\alpha$ -tocopherol levels typically range from one to four  $\alpha$ -tocopherol molecules per 1000 phospholipids (62,63). This range is thought to correspond to an antioxidant threshold for  $\alpha$ -tocopherol (142). Several *in vitro* studies have demonstrated that  $\alpha$ -tocopherol provides effective antioxidant protection only at levels above a threshold concentration (reviewed by Liebler, 143). The  $\alpha$ -tocopherol threshold derives from (1) the tendency of  $\alpha$ -tocopherol to inhibit peroxyl radical propagation and (2) the tendency of peroxyl radicals to consume  $\alpha$ -tocopherol. The threshold essentially represents the  $\alpha$ -tocopherol level at which these two opposing tendencies are in balance. Because membrane  $\alpha$ -tocopherol levels are apparently kept close to a threshold concentration, relatively modest  $\alpha$ -tocopherol depletion could compromise membrane antioxidant defense. Under some circumstances, regeneration of  $\alpha$ -tocopherol from its oxidation products may prevent critical depletion of membrane  $\alpha$ -tocopherol (discussed later). Moreover, *in vitro* studies in a liposome model demonstrated that antioxidant synergism between  $\alpha$ -tocopherol and ascorbate was most efficient when membrane  $\alpha$ -tocopherol levels exceeded the threshold concentration experimentally determined for that system (142). An apparent  $\alpha$ -tocopherol threshold for protection against lipid peroxidation *in vivo* has been deduced from plasma  $\alpha$ -tocopherol levels in rats (144,145).

#### Antioxidant Reactions

TH exerts antioxidant effects primarily by trapping peroxyl radicals [Eqs. (11) and (12)].  $\alpha$ -Tocopherol reacts readily with peroxyl radicals to yield a hydroperoxide and the resonance-stabilized tocopheroxyl radical (T).



The tocopheroxyl radical formed in reaction 11 may be reduced by several biochemical reductants and this is postulated to regenerate  $\alpha$ -tocopherol and complete a one-electron redox cycle (discussed later). Direct observation of reactions 11 and 12 in biological systems generally is not feasible, but much has been learned about  $\alpha$ -tocopherol antioxidant reactions through analyses of the products formed in reaction 12.

Further reactions of the tocopheroxyl radical yield two groups of nonradical products (146–151). The first consists of 8 $\alpha$ -substituted tocopherones, which result either from peroxyl radical addition at C-8 $\alpha$  to form 8 $\alpha$ -(alkyldioxy)tocopherones (1, Figure 3) (146,148,149,152) or from electron transfer followed by hydrolysis to yield 8 $\alpha$ -(hydroxy)tocopherones 2 (151,153,154). Products 1 and 2 hydrolyze and rearrange to form  $\alpha$ -tocopherolquinone 3 (155). Formation of 8 $\alpha$ -substituted tocopherones 1/2 is analogous to reactions of simple antioxidant phenols (e.g., BHT) with peroxyl radicals and is compatible with the previously reported stoichiometry of two peroxyl radicals trapped for each  $\alpha$ -tocopherol oxidized (138,146,156).

The remaining products consist of epoxytocopherones 4/5 and their hydrolysis products 5,6-epoxy- $\alpha$ -tocopherolquinone 6 and 2,3-epoxy- $\alpha$ -tocopherolquinone 7, respectively (149,157). Although the mechanism of  $\alpha$ -tocopherol oxidation to epoxides 4/5 is not known, recent studies of product yield and antioxidant stoichiometry indicated that epoxide product yields vary considerably with reaction environment, but the antioxidant stoichiometry remains essentially unchanged at two radicals scavenged per  $\alpha$ -tocopherol consumed (158,159).

Peroxy radical scavenging by  $\alpha$ -tocopherol thus forms 8 $\alpha$ -substituted tocopherones and epoxytocopherones, which then hydrolyze to more stable  $\alpha$ -tocopherolquinone and epoxyquinone products. In recent studies of the peroxyl radical mediated oxidation of  $\alpha$ -tocopherol in microsomes *in vitro*, tocopherone intermediates were found to account for over half of the  $\alpha$ -tocopherol consumed (160). Mild acid treatment of microsomal incubation samples effected complete conversion of the tocopherone precursors to  $\alpha$ -tocopherolquinone and epoxyquinones (151), which were analyzed by a sensitive stable isotope dilution GC-MS method (160). Analysis of  $\alpha$ -tocopherol, together with  $\alpha$ -tocopherolquinone, its reduction product  $\alpha$ -tocopherolhydroquinone (discussed later), and the epoxyquinones can provide a "snapshot" of the redox distribution of  $\alpha$ -tocopherol and its major oxidation products.

#### Redox Cycles for Vitamin E: One-Electron Redox Cycle

Early observations by Golumbic and Mattill (161) and by Tappel et al. (162) led to the suggestion that  $\alpha$ -tocopherol may be regenerated from its oxidation intermediates by other biochemical reductants and that this redox chemistry would maintain through ongoing oxidative stress. The most widely considered redox cycle



is the one-electron redox cycle in which  $\alpha$ -tocopherol is oxidized by a radical to the tocopheroxyl radical (eq 1), which is then reduced back to  $\alpha$ -tocopherol by a reductant such as ascorbate:



Proof of the chemical feasibility of reaction 13 came from pulse radiolysis work by Packer, Slater, and Willson (163). However, evaluation of this one-electron redox cycle in biological systems has been much more difficult. The large body of work on this problem has been reviewed in detail from different perspectives (143,164-166). Several general observations are presented here.

First, in the numerous reported demonstrations of  $\alpha$ -tocopherol "sparing" by ascorbate, other low-molecular-weight antioxidants, and redox proteins, the extent to which these co-antioxidants acted by regenerating  $\alpha$ -tocopherol or by directly trapping radicals generally was not assessed. This makes it difficult to attribute protection against  $\alpha$ -tocopherol depletion to tocopheroxyl radical recycling *per se* or to independent antioxidant actions of the co-antioxidants. Indeed, it seems likely that both tocopheroxyl radical recycling and direct, co-antioxidant effects of ascorbate may occur to varying degrees in different environments. It is nevertheless noteworthy that in a carefully conducted study of  $\alpha$ -tocopherol turnover in several tissues of the guinea pig, dietary ascorbate status did not measurably affect the kinetics of  $\alpha$ -tocopherol turnover (167).

Second, reduction of the tocopheroxyl radical by ubiquinol, ascorbate, or other co-antioxidants has been unambiguously demonstrated in human low-density lipoproteins (59). These co-antioxidants reverse the novel pro-oxidant effect of the tocopheroxyl radical in the lipoprotein particle by reducing the tocopheroxyl radical to  $\alpha$ -tocopherol and thus "carrying away" the radical from the lipoprotein. In the absence of a coreductant, the tocopheroxyl radical may actually initiate peroxidation of lipoprotein lipid (168).

Third, glutathione and other low-molecular-weight thiols apparently do not reduce the tocopheroxyl radical directly, but instead act to regenerate other antioxidants, such as ascorbate, which then may reduce the tocopheroxyl radical (166,169). Enzymatically mediated synergism between glutathione and  $\alpha$ -tocopherol most likely results from parallel antioxidant actions of glutathione peroxidase enzymes (selenium-dependent glutathione peroxidase, selenium-dependent phospholipid hydroperoxide glutathione peroxidase, and some glutathione *S*-transferases) (38).

### Redox Cycles for Vitamin E: Two-Electron Cycle

Tocopheroxyl radicals that do not undergo a one-electron reduction to  $\alpha$ -tocopherol may instead either disproportionate or react with peroxyl radicals to form a variety of products, as discussed earlier. A large fraction of the products formed are 8a-substituted tocopherones, which may undergo reduction to  $\alpha$ -tocopherol to complete a two-electron redox cycle (151,153-155). The possibility that a two-electron redox

cycle may contribute to  $\alpha$ -tocopherol function has been raised previously and considered in detail recently (143). Although 8a-substituted tocopherones are easily reduced to  $\alpha$ -tocopherol *in vitro* by ascorbate or nordehydroguaiaric acid at acidic pH, it is not clear whether an enzyme-catalyzed reduction also can take place in biological systems. Of interest in this regard are the findings of Chan and colleagues, who described the oxidation of  $\alpha$ -tocopherol in platelets to a product that was converted back to  $\alpha$ -tocopherol by subsequent addition of ascorbate, glutathione, or nordehydroguaiaric acid (170). Although the authors proposed that the reducible intermediate was the tocopheroxyl radical, the conditions of the experiment make it more likely that the intermediates were 8a-substituted tocopherones instead, as we have suggested previously (151). This suggests that a two-electron redox cycle involving 8a-substituted tocopherones could contribute to  $\alpha$ -tocopherol maintenance during oxidative stress.

### $\alpha$ -Tocopherol Esters

Esterified forms of  $\alpha$ -tocopherol are resistant to oxidation and display improved stability over  $\alpha$ -tocopherol. These forms of vitamin E, particularly  $\alpha$ -tocopherol acetate, are frequently used in formulating vitamin supplements and other vitamin E-supplemented products. The esters themselves are inactive as antioxidants. When taken orally,  $\alpha$ -tocopherol esters are efficiently hydrolyzed to the active antioxidant  $\alpha$ -tocopherol by esterases in the gut, and bioavailability as free  $\alpha$ -tocopherol is identical to an equal amount of the unesterified vitamin (171,172). In dosage by other routes, the ester and free  $\alpha$ -tocopherol may not be bioequivalent. For example, topical application of free  $\alpha$ -tocopherol inhibits photocarcinogenesis induced by repeated exposures to UV-B in a mouse model, whereas topical  $\alpha$ -tocopherol acetate is ineffective (173). Polar  $\alpha$ -tocopherol esters (e.g.,  $\alpha$ -tocopherol succinate) have been used in a number of studies *in vitro* to achieve  $\alpha$ -tocopherol supplementation in cell culture systems (136,137,174,175). The water-dispersible hemisuccinate ester confers greater protection against oxidative stress than does either free  $\alpha$ -tocopherol or  $\alpha$ -tocopherol acetate (176,177). This apparently reflects a greater ability of the water-dispersible ester to release  $\alpha$ -tocopherol in proximity to vital locations within cells (178).

### Redox Chemistry of $\alpha$ -Tocopherolquinone

$\alpha$ -Tocopherolquinone (8, Figure 3), a stable end-product of  $\alpha$ -tocopherol oxidation (discussed earlier) undergoes facile two-electron reduction by ascorbate, sodium borohydride, or other reductants to  $\alpha$ -tocopherolhydroquinone at neutral pH (179,180). This hydroquinone would be expected to exert antioxidant effects similar to those of ubiquinols, which have been shown to be effective chain-breaking antioxidants. Hayashi et al. (181) demonstrated that isolated rat hepatocytes contained both  $\alpha$ -tocopherolquinone and  $\alpha$ -tocopherolhydroquinone in comparable

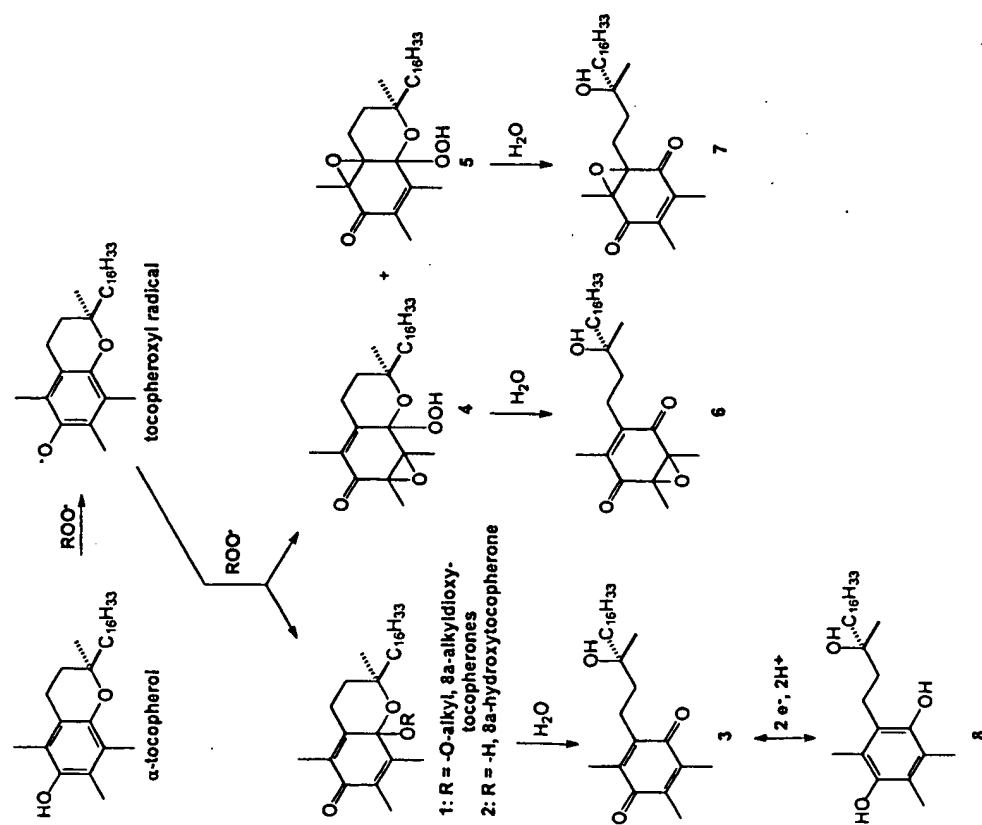


FIG. 3. Reaction products formed during the trapping of peroxyl radicals with  $\alpha$ -tocopherol.

amounts and that the cells were capable of rapidly reducing exogenously added  $\alpha$ -tocopherolquinone to the hydroquinone. Relatively little is known about the fate and possible role of  $\alpha$ -tocopherolquinone formed by oxidative  $\alpha$ -tocopherol turnover. Reduction of the quinone to the hydroquinone could provide an important contribution to cellular antioxidant protection, as proposed recently by Kohar et al. (182).

#### Antioxidant Protection by Vitamin C

In addition to its probable participation in the recycling of  $\alpha$ -tocopherol (discussed earlier), ascorbate is thought to exert direct antioxidant effects. This follows from

the high reactivity of ascorbate as a one-electron reductant for many biologically relevant oxidants (16). The ability of ascorbate to exert both pro-oxidant and antioxidant effects complicates interpretations of its role as a cellular protectant. Antioxidant effects may be due either to (1) direct reaction with free radicals or nonradical oxidants to produce less reactive products (see Frei, 183, for a recent review), (2) regeneration of phenolic antioxidants such as  $\alpha$ -tocopherol (discussed earlier), or (3) shifting the redox balance of transition metal redox couples to disfavor their participation in pro-oxidant reactions (184). As discussed earlier, some combination of the first two mechanisms probably accounts for antioxidant synergy between ascorbate and  $\alpha$ -tocopherol. In most experimental systems, it may not be possible to distinguish the relative contributions of these mechanisms. Pro-oxidant effects of ascorbate are often observed in *in vitro* systems and are thought to be due to (1) reduction of transition metal ions to facilitate participation in Fenton chemistry (see Koppenol, chapter 1, this volume) and (2) formation of reactive oxygen species subsequent to metal catalyzed ascorbate autooxidation. It seems clear that ascorbate can inhibit or enhance metal catalyzed oxidations, depending on the ascorbate concentration (184).

The balance between pro-oxidant and antioxidant effects of ascorbate also may be controlled by the membrane status of  $\alpha$ -tocopherol. In studies with a liposome model, in which oxidation was initiated by  $\text{Fe}^{2+}$  and hydrogen peroxide, ascorbate alone at concentrations of less than 1 mM exerted a marked pro-oxidant effect, apparently by contributing to Fenton chemistry (142). Inclusion of  $\alpha$ -tocopherol at a concentration of 0.2 mol%, which is above the  $\alpha$ -tocopherol antioxidant threshold for that system, reversed the pro-oxidant effect of ascorbate. This coincided with an ascorbate-dependent prevention of  $\alpha$ -tocopherol depletion. In liposomes containing  $\alpha$ -tocopherol at lower concentrations, ascorbate was unable either to prevent  $\alpha$ -tocopherol depletion or to prevent lipid peroxidation.

Intracellular ascorbate is consumed and recycled by permeant oxidants in activated neutrophils (185). Since free-radical generation by neutrophils can be as high as 200 nmole/ $10^6$  cells/h (10), recycling of ascorbate occurs with rapid reduction of dehydroascorbate by a yet unknown mechanism for reduction. The accumulation of ascorbate and dehydroascorbate occurs by separate mechanisms (11). Winkler et al. (186) recently reviewed the literature on mechanisms for ascorbate reduction in cells. They concluded that there is little convincing evidence for the existence of putative NADH-dependent semidehydroascorbate reductases in mammalian cells, despite the fact that these enzymes are well described in plants. These authors concluded that semidehydroascorbate formed by one-electron oxidations of ascorbate disproportionates to dehydroascorbic acid, which then either is reduced nonenzymatically by glutathione or hydrolyzes to nonreducible products. Other mechanisms may maintain reduced ascorbate, including protein disulfide isomerase and glutaredoxin, which have dehydroascorbate-reducing activity (187) that is dependent on reduced glutathione. Welch et al. (11) have shown that in neutrophils dehydroascorbate reduction is protein mediated and chemical reduction by GSH could not account for the reduction. Also, they have found that dehydroascorbate transport and accumulation is 10-fold greater than for ascorbate. These findings support the concept of

extracellular defense against free radicals and other oxidants being an important aspect of the utilization and recycling of ascorbate, with intracellular reducing equivalents being made readily available by a glutathione-dependent reduction process (188).

## CONCLUSIONS

Over the past two decades, a large body of work has helped to explain the functions of biological antioxidants and antioxidant enzyme systems. What has more recently emerged is a broader picture of the integration of biological antioxidant defense. Just as biological oxidant challenges encompass a diverse array of primordial oxidants, secondary oxidants, and propagating radicals, so do biological antioxidants comprise a multitiered defense. Specialization in antioxidant function allows specific enzymes and small molecules to scavenge specific oxidants with high efficiency. A diverse antioxidant defense system permits cells to defend against multiple components of oxidant challenges.

In a review of antioxidant therapy, Rice-Evans and Diplock (141) chose to concentrate mainly on coronary heart disease, reperfusion injury, and organ storage for transplantation. They provided ample evidence for free radicals having a major contribution to these conditions and insight on how antioxidant therapy could be beneficial. However, as they point out, "only when the mechanisms and involvement of radicals in the pathogenesis of many disorders described in this review are understood will approaches to antioxidant therapies be designed effectively and targeted successfully." We agree with these comments and we think that they apply equally well to understanding how antioxidant defense affects injury by toxic chemicals. We hope that this review offers perspectives on the functions of cellular antioxidant defense that will prove useful to those investigating chemically induced tissue injury and free-radical-associated diseases.

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## REFERENCES

1. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: An overview. In: Packer L, and Glazer A.N., eds. *Methods in Enzymology*. San Diego: Academic Press, 1990;186(B):1-85.

2. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 1979;59:527-605.
3. Cadenas E. Biochemistry of oxygen toxicity. *Annu Rev Biochem* 1989;58:79-110.
4. Richter C. Do mitochondrial DNA fragments promote cancer and aging? *FEBS Lett* 1988;241:1-5.
5. Forman HJ, Boveris A. Superoxide radical and hydrogen peroxide in mitochondria. In: Pryor WA, ed., *Free Radicals in Biology*. Vol V, pp. 65-90. New York: Academic Press, 1982.
6. Jones DP, Eklow L, Thor H, Orrenius S. Metabolism of hydrogen peroxide in isolated hepatocytes: Relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated  $H_2O_2$ . *Arch Biochem Biophys* 1981;210:505-516.
7. Sohal RS. Aging, cytochrome oxidase activity, and hydrogen peroxide release by mitochondria. *Free Radical Biol Med* 1993;14:583-588.
8. Turrens JF, McCord JM. Mitochondrial generation of reactive oxygen species. In: Paulet AC, Douste-Blazy L, Paoletti R, eds. *Free Radicals, Lipoproteins, and Membrane Lipids*. New York: Plenum Press, 1990:203-212.
9. Boveris A, Cadenas E. Production of superoxide in mitochondria. In: Oberley, L.W., ed., *Superoxide Dismutase, II*. Boca Raton, FL: CRC Press, 1982;15-30.
10. Mehlforn RJ. Ascorbate and dehydroascorbic acid-mediated reduction of free radicals in the human erythrocyte. *J Biol Chem* 1991;266:2724-2731.
11. Welch RW, Wang Y, Crossman Jr A, Park JB, Kirks KL and Levine M. Accumulation of vitamin C (ascorbate) and its oxidized metabolite dehydroascorbic acid occurs by separate mechanisms. *J Biol Chem* 1995;270:12585-12592.
12. Babior BM. Oxidant from phagocytes: Agents of defense and destruction. *Blood* 1984;64:959-966.
13. Babior BM. The respiratory burst oxidase. *Trends Biol Sci* 1987;12:241-242.
14. Frenkel K. Carcinogen-mediated oxidant formation and oxidative DNA damage. *Pharmacol Ther* 1992;53:127-166.
15. Morris SM Jr, Billiar TR. New insights into the regulation of inducible nitric oxide synthesis. *Am J Physiol* 1994;266:E829-E839.
16. Beutner GR. The pecking order of free radicals and antioxidants: Lipid peroxidation,  $\alpha$ -tocopherol, and ascorbate. *Arch Biochem Biophys* 1993;300:535-543.
17. Beckman JS, Crow JP. Pathological implications of nitric oxide, superoxide, and peroxynitrite formation. *Biochem Soc Trans* 1993;21:330-334.
18. Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H, Beckman JS. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem Res Toxicol* 1992;5:834-842.
19. Pryor WA, Lightsey JW. Mechanisms of nitrogen dioxide reactions: Initiation of lipid peroxidation and the production of nitrous acid. *Science* 1981;214:435-437.
20. Svingen BA, Beuge JA, O'Neal FO, Aust SD. The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. *J Biol Chem* 1979;254:5892-5899.
21. Aikens J, Dix TA. Hydrodioxy (perhydroxyl), peroxyl, and hydroxyl radical-initiated lipid peroxidation of large unilamellar vesicles (liposomes): Comparative and mechanistic studies. *Arch Biochem Biophys* 1993;305:516-525.
22. Wilcox AL, Marnett LJ. Polyunsaturated fatty acid alkoxyl radicals exist as carbon-centered epoxyallylic radicals: A key step in hydroperoxide-amplified lipid peroxidation. *Chem Res Toxicol* 1993;6:413-416.
23. Wardman P. Conjugation and oxidation of glutathione via thiol free radicals. In: Sies H, Ketterer B, eds. *Glutathione Conjugation: Mechanisms and Biological Significance*. London: Academic Press, 1988;44-72.
24. Pryor WA. Free radicals in autoxidation and aging. In: Armstrong D, Sohal RS, Cutler RG, Slater TF, eds., *Free Radicals in Molecular Biology, Aging, and Disease*. New York: Raven Press, 1984;13-41.
25. Fridovich I. Superoxide dismutases. *Adv Enzymol Relat Areas Mol Biol* 1974;41:35-97.
26. Fridovich I. Superoxide dismutase. An adaptation to a paramagnetic gas. *J Biol Chem* 1989;264:7761-7764.
27. Marklund SL. Human copper-containing superoxide dismutase of high molecular weight. *Proc Natl Acad Sci USA* 1982;79:7634-7638.
28. Wong GHW. Protective roles of cytokines against radicalization: Induction of mitochondrial MnSOD. *Biochim Biophys Acta* 1995;1271:205-209.
29. Shull S, Heintz NH, Periasamy M, Manohar M, Janssen YMW, Marsh JP, Mossman BT. Differential regulation of antioxidant enzymes in response to oxidants. *J Biol Chem* 1991;266:24398-24403.

0. Chu FF, Doroshov JH, Esworthy RS. Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. *J Biol Chem* 1993;268:2571-2576.
1. Wendel A. Glutathione peroxidase. In: Jakoby WB, ed. *Enzymatic Basis of Detoxication*. New York: Academic Press, 1980;333-354.
2. Takahashi K, Akasaka M, Yamamoto Y, Kobayashi J, Mizoguchi J, Koyama J. Primary structure of human plasma glutathione peroxidase deduced from cDNA sequences. *J Biochem* 1990;108:145-148.
3. Ursini F, Maiorino M, Gregolin C. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochim Biophys Acta* 1985;839:62-70.
4. Ursini F, Maiorino M, Valente M, Ferri L, Gregolin C. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochim Biophys Acta* 1982;710:197-211.
5. Nakamura W, Hosoda S, Hayashi K. Purification and properties of rat liver glutathione peroxidase. *Biochim Biophys Acta* 1974;358:251-261.
6. Timmerstein MA, Reed DJ. Effects of glutathione on the  $\alpha$ -tocopherol-dependent inhibition of nuclear lipid peroxidation. *J Lipid Res* 1989;30:959-965.
7. Burk RF, Hill KE. Selenoprotein P. A selenium-rich extracellular glycoprotein. *J Nutr* 1994;124:1891-1897.
8. Maiorino M, Coassin M, Roveri A, Ursini F. Microsomal lipid peroxidation: Effect of vitamin E and its functional interaction with phospholipid hydroperoxide glutathione peroxidase. *Lipids* 1989;24:721-726.
9. Weitzel F, Wendel A. Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. *J Biol Chem* 1992;268:6288-6292.
10. Awad JA, Morrow JD, Hill KE, Roberts LJII, Burk RF. Detection and localization of lipid peroxidation in selenium- and vitamin E-deficient rats using F<sub>2</sub>-isoprostanes. *J Nutr* 1994;124:810-816.
11. Michiels C, Raes M, Toussaint O, Remele J. Importance of Se-glutathione peroxidase, catalase and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radical Biol Med* 1994;17:235-248.
12. Margolish E, Novogrodsky A, Schejter A. Irreversible reaction of 3-amino-1,2,4-triazole and related inhibitors with the protein of catalase. *Biochem J* 1960;74:339-348.
13. Heikkilä RE, Cabat FS, Cohen G. In vivo inhibition of superoxide dismutase in mice by diethylthiocarbamate. *J Biol Chem* 1976;252:2182-2185.
14. Chaudiere J, Wilhelm EC, Tappel AL. Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptopyruvic acid and other mercaptans. *J Biol Chem* 1984;259:1043-1050.
15. Babson JR, Reed DJ. Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanates. *Biochem Biophys Acta* 1978;53:754-762.
16. Griffith OW. Mechanism of action, metabolism and toxicity of buthionine sulfoximine and its higher analogs, potent inhibitors of glutathione synthesis. *J Biol Chem* 1982;257:3704-3712.
17. Wispe JR, Warner BB, Clark JC, Dey CR, Neuman J, Glasser SW, Crapo JD, Chang L-Y, Whitsett JA. Human Mn-superoxide dismutase in pulmonary epithelial cells of transgenic mice confers protection from oxygen injury. *J Biol Chem* 1992;267:23937-23941.
18. St. Clair DK, Oberley TD, Ho Y-S, Wheeler KT. Overproduction of human MnSOD modulates paraquat-mediated toxicity in mammalian cells. *FEBS Lett* 1991;293:199-203.
19. Scott MD, Meshnick SR, Eaton JW. Superoxide dismutase-rich bacteria. Paradoxical increase in oxidant toxicity. *J Biol Chem* 1987;262:3640-3645.
20. Liochev SI, Fridovich I. Effects of overproduction of superoxide dismutase on the toxicity of paraquat toward *Escherichia coli*. *J Biol Chem* 1991;266:8747-8750.
21. Warner HR. Superoxide dismutase, aging, and degenerative disease. *Free Radical Biol Med* 1994;17:249-258.
22. Luo Y, Han Z, Chin SM, Linn S. Three chemically distinct types of oxidants formed by iron-mediated Fenton reactions in the presence of DNA. *Proc Natl Acad Sci USA* 1994;91:12438-42.
23. Krinsky NI. Actions of carotenoids in biological systems. *Annu Rev Nutr* 1993;13:561-587.
24. Liebler DC. Antioxidant reactions of carotenoids. In: Canfield LM, Krinsky NI, Olson JA, eds., *Carotenoids in Human Health. Annals of the New York Academy of Sciences*. New York: New York Academy of Sciences, 1993;20-31.
25. Packer L, Witt EH, Trischie HJ. Alpha-lipoic acid as a biological antioxidant. *Free Radical Biol Med* 1995;19:227-250.

56. Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic MG. Flavonoids as antioxidants. *J Am Chem Soc* 1994;116:4846-4851.
57. Terao J, Piskula M, Yao Q. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Arch Biochem Biophys* 1994;308:278-284.
58. Kagan VE, Serbinova EA, Koyanova GM, Kitanova SA, Tyurin VA, Solychev TS, Quinn PJ, Packer L. Antioxidant action of ubiquinol homologues with different isoprenoid chain length in biomembranes. *Free Radical Biol Med* 1990;9:117-126.
59. Bowry VW, Mohr D, Cleary J, Stocker R. Prevention of tocopherol-mediated peroxidation in ubiquinol-10-free human low density lipoprotein. *J Biol Chem* 1995;270:5756-5763.
60. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science* 1987;235:1043-1046.
61. Simic MG, Jovanovic SV. Antioxidation mechanisms of uric acid. *J Am Chem Soc* 1989;111:5778-5782.
62. Kornbrust DJ, Mavis RD. Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: correlation with vitamin E content. *Lipids* 1980;15:315-322.
63. evanian A, Hacker AD, Elsayed N. Influence of vitamin E and nitrogen dioxide on lipid peroxidation in rat lung and liver microsomes. *Lipids* 1982;17:269-277.
64. Howard JA, Ingold KU. Absolute rate constants for hydrocarbon autoxidation. VI. Alkyl aromatic and olefinic hydrocarbons. *Can J Chem* 1967;45:793-802.
65. Iwasaki M, Tsuchiya J, Komuro E, Yamamoto Y, Niki E. Effects of solvents and media on the antioxidant activity of  $\alpha$ -tocopherol. *Biochim Biophys Acta* 1994;1200:19-26.
66. Czapski G. Reaction of OH. *Methods Enzymol* 1984;105:209-215.
67. Simic MG. Antioxidant compounds: An overview. In: Davies KJA, eds. *Oxidative Damage and Repair. Chemical, Biological, and Medical Aspects*. Oxford: Pergamon Press, 1991;47-56.
68. Burton GW, Ingold KU. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc Chem Res* 1986;19:194-201.
69. Hill TJ, Land EJ, McGarvey DJ, Schalch W, Tinkler JH, Truscott TG. Interactions between carotenoids and the  $\text{CCl}_3\text{O}_2$  radical. *J Am Chem Soc* 1993;117:8322-8326.
70. Liebler DC, McClure TD. Antioxidant reactions of  $\beta$ -carotene: Identification of carotenoid-radical adducts. *Chem Res Toxicol* 1996; 9:8-11.
71. Kosower NS, Kosower EM. Glutathione status of cells. *Int Rev Cytol* 1978;54:109-160.
72. Reed DJ, Beatty PW. Biosynthesis and regulation of glutathione. Toxicological implications. In: Hodgson E, Bend JR, Philpot RM, eds. *Reviews in Biochemical Toxicology*. New York: Elsevier Press, 1980;213-241.
73. Reed DJ. Glutathione: Toxicological implications. *Annu Rev Pharmacol Toxicol* 1990;30:603-631.
74. Reed DJ. Cellular defense mechanisms against reactive metabolites. In: Anders MW, ed. *Bioactivation of Foreign Compounds*. Orlando FL: Academic Press, 1985;71-108.
75. Reed DJ. Toxicity of Oxygen. In: De Matteis F, Smith LL, eds. *Molecular and Cellular Mechanisms of Toxicity*. Boca Raton, FL: CRC Press, 1995;35-68.
76. White INH. Role of liver glutathione in acute toxicity of retrorsine to rats. *Chem Biol Interact* 1976;13:333-342.
77. Tateishi N, Higashi T, Naruse A, et al. Rat-liver glutathione. Possible role as a reservoir of cysteine. *J Nutr* 1977;107:51-60.
78. Higashi T, Tateishi N, Naruse A, et al. Novel physiological role of liver glutathione as a reservoir of L-cysteine. *J Biochem* 1977;82:117-124.
79. Cho ES, Sahyoun N, Stegink LD. Tissue glutathione as a cyst(e)ine reservoir during fasting and refeeding of rats. *J Nutr* 1981;111:914-922.
80. Meredith MJ, Reed DJ. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J Biol Chem* 1982;257:3747-3753.
81. Womack M, Kremer KS, Rose WC. The relation of cysteine and methionine to growth. *J Biol Chem* 1937;121:403-410.
- 82a. Reed DJ, Ellis WW. Influence of  $\gamma$ -glutamyl transpeptidase inactivation on the status of extracellular glutathione and glutathione conjugates. In: Snyder R, Parke CV, Kocsis JJ, et al., eds. *Biological Reactive Intermediates, IIA*. New York: Plenum Press, 1982;75-86.
- 82b. Meister A. Glutathione-ascorbic acid antioxidant system in animals. *J Biol Chem* 1994; 269:9397-9400.
83. Younes M, Siegers C-P. Mechanistic aspects of enhanced lipid peroxidation following glutathione depletion in vivo. *Chem Biol Interact* 1981;34:257-266.

84. Brunk UT, Jones CB, Sohal RS. A novel hypothesis of lipofuscinogenesis and cellular ageing based on interactions between oxidative stress and autophagocytosis. *Mutat Res* 1992;275:395-403.
85. Gao G, Ollinger K, Brunk UT. Influence of intracellular glutathione concentration of lipofuscin accumulation in cultured neonatal rat cardiac myocytes. *Free Radical Biol Med* 1994;16:187-194.
86. Reed DJ. Regulation of reductive processes by glutathione. *Biochem Pharmacol* 1986;35:7-13.
87. Lopez-Barea J, Lee C-Y. Mouse-liver glutathione reductase. Purification, kinetics, and regulation. *Eur J Biochem* 1979;98:487-499.
88. Bachur NR, Gordon SL, Gee MV. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res* 1978;38:1745-1750.
89. Babson JR, Abell NS, Reed DJ. Protective role of the glutathione redox cycle against adriamycin-mediated toxicity in isolated hepatocytes. *Biochem Pharmacol* 1981;30:2299-2304.
90. Meredith MJ, Reed DJ. Depletion *in vitro* of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem Pharmacol* 1983;32:1383-1388.
91. Christophersen BO. Formation of monohydroxy-polyenic fatty acids from lipid peroxides by a glutathione peroxidase. *Biochim Biophys Acta* 1968;164:35-46.
92. McCay PB, Lai EK, Powell SR, et al. Vitamin E functions as an electron shuttle for glutathione-dependent "free radical reductase" activity in biological membranes. *Fed Proc Fed Am Soc Exp Biol* 1976;45:1729.
93. Yonaha M, Tampo Y. Bromosulphophthalen abolishes glutathione-dependent protection against lipid peroxidation in rat liver mitochondria. *Biochem Pharmacol* 1987;36:2831-2837.
94. Tirmerstein MA, Reed DJ. Characterization of glutathione-dependent inhibition of lipid peroxidation of isolated rat liver nuclei. *Arch Biochem Biophys* 1988;261:1-11.
95. McCay PB, Brueggemann G, Lai EK, Powell SR. Evidence that  $\alpha$ -tocopherol functions cyclically to quench free radicals in hepatic microsomes. Requirement for glutathione and a heat-labile factor. *Ann NY Acad Sci* 1989;570:32-45.
96. Reddy CC, Sholz WW, Thomas CB, et al. Vitamin E-dependent reduced glutathione inhibition of rat liver microsomal lipid peroxidation. *Life Sci* 1982;31:571-576.
97. Burk RF. Glutathione-dependent protection by rat liver microsomal protein against lipid peroxidation. *Biochim Biophys Acta* 1983;757:21-28.
98. Burk RF, Trumble KR, Lawrence RA. Rat hepatic cytosolic glutathione-dependent enzyme protection against lipid peroxidation in the NADPH-microsomal lipid peroxidation system. *Biochim Biophys Acta* 1980;618:35-41.
99. Haenen GRMM, Bast A. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. *FEBS Lett* 1983;159:24-28.
100. Akasaka S. Inactivation of transforming activity of plasmid DNA by lipid peroxidation. *Biochim Biophys Acta* 1986;867:201-208.
101. Gibson DD, Hawrylko J, McCay P B. GSH-dependent inhibition of lipid peroxidation: Properties of a potent cytosolic system which protects cell membranes. *Lipids* 1985;20:704-711.
102. Takayanagi R, Takeshige K, Minakami S. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxidant role of ubiquinol. *Biochem J* 1980;192:853-860.
103. Bindoli A, Cavallini L, Jocelyn P. Mitochondrial lipid peroxidation by cumene hydroperoxide and its prevention by succinate. *Biochim Biophys Acta* 1982;681:496-503.
104. Mészáros L, Tihanyi K, Horvath I. Mitochondrial substrate oxidation-dependent protection against lipid peroxidation. *Biochim Biophys Acta* 1982;713:675-677.
105. Flohé L, Zimmermann R. The role of GSH peroxidase in protecting the membrane of rat liver mitochondria. *Biochim Biophys Acta* 1970;223:210-213.
106. McCay P B, Lai EK, Powell SR, et al. Vitamin E functions as an electron shuttle for glutathione-dependent "free radical reductase" activity in biological membranes. *Fed Proc Fed Am Soc Exp Biol* 1986;45:1729.
107. Kraus P. Resolution, purification and some properties of three glutathione transferases from rat liver mitochondria. *Hoppe-Seyler's Z Physiol Chem* 1980;361:9-15.
108. Morgenstern R, Lundqvist G, Andersson G, et al. The distribution of microsomal glutathione transferase among different organelles, different organs, and different organisms. *Biochem Pharmacol* 1984;33:3609-3614.
109. Franke WW. Structure, biochemistry, and functions of the nuclear envelope. *Int Rev Cytol Suppl* 1974;4:71-236.
110. Gibson DD, Hornbrook KR, McCay PB. Glutathione-dependent inhibition of lipid peroxidation by a soluble, heat-labile factor in animal tissues. *Biochim Biophys Acta* 1980;620:572-582.
111. Ueda K, Kobayashi S, Morita J, Komano T. Site-specific DNA damage caused by lipid peroxidation products. *Biochim Biophys Acta* 1984;824:341-348.
112. Brawn K, Fridovich I. DNA strand scission by enzymically generated oxygen radicals. *Arch Biochem Biophys* 1981;206:414-419.
113. Mukai FH, Goldstein BD. Mutagenicity of malonaldehyde, a decomposition product of peroxidized polyunsaturated fatty acids. *Science* 1976;191:868-869.
114. Roots R, Okada S. Estimation of life times and diffusion distances of radicals involved in x-ray-induced DNA strand breaks of killing of mammalian cells. *Radiat Res* 1975;64:306-320.
115. Shigenaga MK, Ames BN. Assays for 8-hydroxy-2'-deoxyguanosine: A biomarker of *in vivo* oxidative DNA damage. *Free Radical Biol Med* 1991;10:211-216.
116. Hjelte JJ, Petersen DR. Metabolism of malondialdehyde by rat liver aldehyde dehydrogenase. *Toxicol Appl Pharmacol* 1983;70:57-66.
117. Alin P, Danielson UH, Mannervik B. 4-Hydroxyalk-2-enals are substrates for glutathione transferase. *FEBS Lett* 1985;179:267-270.
118. Gruger EH, Tappel AL. Reactions of biological antioxidants: III. Composition of biological membranes. *Lipids* 1971;6:147-148.
119. Jocelyn PC. Some properties of mitochondrial glutathione. *Biochim Biophys Acta* 1975;396:427-436.
120. Kurosawa K, Hayashi N, Sato N, Kamada T, Tagawa K. Transport of glutathione across mitochondrial membranes. *Biochim Biophys Res Commun* 1990;167:367-372.
121. Garcia-Ruiz C, Morales A, Collell A, Rodas J, Yi J, Kaplowitz N, Fernandez-Checa J. Evidence that the rat hepatic mitochondrial carrier is distinct from the sinusoidal and canalicular transporter for reduced glutathione: Expression studies in *Xenopus laevis* oocytes. *J Biol Chem* 1995;270:15946-15949.
122. Wahländer A, Sobell S, Sies H, Linke I, Müller M. Hepatic mitochondrial and cytosolic glutathione content and the subcellular distribution of GSH-S-transferase. *FEBS Lett* 1979;97:138-140.
123. Olafsdottir K, Reed DJ. Retention of oxidized glutathione by isolated rat liver mitochondria during hydroperoxide treatment. *Biochim Biophys Acta* 1988;377-382.
124. Shan X, Jones DP, Hashmi M, Anders MW. Selective depletion of mitochondrial glutathione concentrations by (R,S)-3-hydroxy-4-pentenolone potentiates oxidative cell death. *Chem Res Toxicol* 1993;6:75-82.
125. Yen H-C, Oberley TD, Ho Y-S, Vichitbandha S, St. Clair DK. An antioxidant protective mechanism against adriamycin-induced cardiac toxicity. In: *Oxygen '95, 1995; The Annual Meeting of the Oxygen Society*, 1995:58.
126. Flohé L, Schlegel W. Glutathione peroxidase. IV. Intracellular distribution of the glutathione peroxidase system in the rat liver. *Hoppe-Seyler's Z Physiol Chem* 1971;352:1401-1410.
127. Neubert D, Wojtysak AB, Lehninger AL. Purification and enzymatic identity of mitochondrial contraction factors I and II. *Proc Natl Acad Sci USA* 1962;48:1651-1658.
128. Vignais PM, Vignais PV. Fusicin, an inhibitor of mitochondrial SH-dependent transport-linked functions. *Biochim Biophys Acta* 1973;325:357-374.
129. Sies H, Moss KM. A role of mitochondrial glutathione peroxidase in modulating mitochondrial oxidations in liver. *Eur J Biochem* 1978;84:377-383.
130. Moore G A, O'Brien PJ, Orenius S. Menadione (2-methyl-1,4-naphthoquinone)-induced  $\text{Ca}^{2+}$  release from rat-liver mitochondria is caused by NAD(P)H oxidation. *Xenobiotica* 1986;16:873-882.
131. Smith PF, Alberts DW, Rush GF. Role of glutathione reductase during menadione-induced NADPH oxidation in isolated rat hepatocytes. *Biochem Pharmacol* 1987;36:3879-3884.
132. Webb JL. Sulfhydryl reagents (Chapter 4), Mercurials (Chapter 7). In: Webb JL, ed. *Enzyme and Metabolic Inhibitors*, Vol. 2. New York: Academic Press, 1965.
133. Webb JL. Iodoacetate and iodoacetamide (Chapter 1), N-Ethylmaleimide (Chapter 3), Arsenicals (Chapter 6), Comparison of SH reagents (Chapter 7). In: Webb JL, ed. *Enzyme and Metabolic Inhibitors*, Vol. 3. New York: Academic Press, 1966.
134. Dean RT, Cheesman KH. Vitamin E protects against free radical damage in lipid environments. *Biochim Biophys Res Commun* 1987;148:1277-1282.
135. Akerboom TPM, Bilzer M, Sies H. The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. *J Biol Chem* 1982;257:4248-4252.

136. Pascoe G A, Reed DJ. Relationship between cellular calcium and vitamin E metabolism during protection against cell injury. *Arch Biochem Biophys* 1987;253:287-296.
137. Pascoe GA, Reed DJ. Vitamin E protection against chemical-induced injury. II. Evidence for a threshold effect in prevention of adriamycin toxicity. *Arch Biochem Biophys* 1987;256:159-166.
138. Burton GW, Ingold KU. Autoxidation of biological molecules. I. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *J Am Chem Soc* 1981;103:6472-6477.
139. Chow CK, Vitamin E and oxidative stress. *Free Radical Biol Med* 1991;11:215-232.
140. Diplock AT. Antioxidant nutrients and disease prevention: An overview. *Am J Clin Nutr* 1991;53:189S-193S.
141. Rice-Evans CA, Diplock AT. Current status of antioxidant therapy. *Free Radical Biol Med* 1993;15:77-96.
142. Liebler DC, Kling DS, Reed DJ. Antioxidant protection of phospholipid bilayers by  $\alpha$ -tocopherol. *J Biol Chem* 1986;261:12114-12119.
143. Liebler DC. The role of metabolism in the antioxidant function of vitamin E. *Crit Rev Toxicol* 1993;23:147-169.
144. Litov RE, Matthews LC, Tappel AL. Vitamin E protection against in vivo lipid peroxidation initiated in rats by methyl ethyl ketone peroxide as monitored by pentane. *Toxicol Appl Pharmacol* 1981;59:96-106.
145. Herschberger LA, Tappel AL. Effect of vitamin E on pentane exhaled by rats treated with methyl ethyl ketone peroxide. *Lipids* 1982;17:686-691.
146. Winerle J, Dulin D, Mill T. Products and stoichiometry of reaction of vitamin E with alkylperoxy radicals. *J Org Chem* 1984;49:491-495.
147. Matsuo M, Matsumoto S, Iitaka Y, Kato K, Ueno Y. Reaction products of  $\alpha$ -tocopherol with a model compound, 2,2,5,7,8-pentamethylchroman-6-ol, in a *tert*-butylperoxy radical generating system. *J Am Chem Soc* 1989;111:1719-1785.
148. Yamauchi R, Matsui T, Sakae Y, Kato K, Ueno Y. Reaction products of  $\alpha$ -tocopherol with a free radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile). *Lipids* 1989;24:204-209.
149. Liebler DC, Baker PF, Kayser KL. Oxidation of vitamin E: Evidence for competing autoxidation and peroxyl radical trapping reactions of the tocopheroxyl radical. *J Am Chem Soc* 1990;112:6995-7000.
150. Yamauchi R, Matsui T, Kato K, Ueno Y. Reaction products of  $\alpha$ -tocopherol with methyl linoleate-peroxyl radicals. *Lipids* 1990;25:152-158.
151. Liebler DC, Burr JA. Oxidation of vitamin E during iron-catalyzed lipid peroxidation: Evidence for electron-transfer reactions of the tocopheroxyl radical. *Biochemistry* 1992;31:8278-8284.
152. Yamauchi R, Yagi Y, Kato K. Isolation and characterization of addition products of  $\alpha$ -tocopherol with peroxyl radicals of dlinoleoylphosphatidylcholine in liposomes. *Biochim Biophys Acta* 1994;1212:43-49.
153. Durrheimer W, Cohen LA. The chemistry of 9-hydroxy- $\alpha$ -tocopherone, a quinone hemiacetal. *J Am Chem Soc* 1964;86:4388-4393.
154. Marcus MF, Hawley MD. Electrochemical studies of the redox behavior of  $\alpha$ -tocopherol. *Biochim Biophys Acta* 1970;201:1-8.
155. Liebler DC, Kayser KL, Kennedy TA. Redox cycles of vitamin E: Hydrolysis and ascorbic acid dependent reduction of 8-alkyldioxytocopherones. *Biochemistry* 1989;28:9772-9777.
156. Niki E, Tanimura R, Kamiya Y. Oxidation of lipids. II. Rate of inhibition of oxidation by  $\alpha$ -tocopherol and hindered phenols measured by chemiluminescence. *Bull Chem Soc Jpn* 1982;55:1551-1555.
157. Liebler DC, Kayser KL, Burr JA. Peroxyl radical trapping and autoxidation reactions of  $\alpha$ -tocopherol in lipid bilayers. *Chem Res Toxicol* 1991;4:89-93.
158. Liebler DC, Burr JA, Matsumoto S, Matsuo M. Reactions of the vitamin E model compound 2,2,5,7,8-pentamethylchroman-6-ol with peroxyl radicals. *Chem Res Toxicol* 1993;6:351-355.
159. Liebler DC and Burr JA. Antioxidant stoichiometry and the oxidative fate of vitamin E in peroxyl radical scavenging reactions. *Lipids* 1995;30:789-793.
160. Liebler DC, Burr JA, Phillips L, Ham AJL. Gas chromatography-mass spectrometry analysis of vitamin E and its oxidation products. *Anal Biochem* 1996;236:27-34.
161. Golumbic C, Mattill HA. Antioxidants and the autoxidation of fats. XIII. The antioxygenic action of ascorbic acid in association with tocopherols, hydroquinones and related compounds. *J Am Chem Soc* 1941;63:1279-1280.

162. Tappel AL, Brown WD, Zalkin H, Maier VP. Unsaturated lipid peroxidation catalyzed by hematin compounds and its inhibition by vitamin E. *J Am Oil Chem Soc* 1961;38:5-9.
163. Packer JE, Slater TF, Willson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* 1979;278:737-738.
164. McCay PB. Vitamin E: Interactions with free radicals and ascorbate. *Annu Rev Nutr* 1985;5:323-340.
165. Niki E. Interaction of ascorbate and  $\alpha$ -tocopherol. *Am NY Acad Sci* 1987;498:186-199.
166. Packer L, Kagan, VE. Vitamin E: The antioxidant harvesting center of membranes and lipoproteins. In: Fuchs J, Packer L, eds. *Vitamin E in Health and Disease*. New York: Marcel Dekker, 1993;179-192.
167. Burton GW, Traber MG. Vitamin E: Antioxidant activity, biokinetics, and bioavailability. *Annu Rev Nutr* 1990;10:357-382.
168. Bowry VW, Stocker R. Tocopherol-mediated peroxidation. The pro-oxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J Am Chem Soc* 1993;115:6029-6044.
169. Barclay LRC. The cooperative antioxidant role of glutathione with a lipid-soluble and a water-soluble antioxidant during peroxidation of liposomes initiated in the aqueous phase and in the lipid phase. *J Biol Chem* 1988;263:16138-16142.
170. Chan AC, Tran K, Raynor T, Garz PR, Chow CK. Regeneration of vitamin E in human platelets. *J Biol Chem* 1991;266:17290-17295.
171. Burton GW, Ingold KU, Foster DO. Comparison of free  $\alpha$ -tocopherol and  $\alpha$ -tocopherol acetate as sources of vitamin E in rats and humans. *Lipids* 1988;23:834-840.
172. Burton GW, Traber MG. Vitamin E antioxidant activity, biokinetics and bioavailability. *Ann. Rev. Nutrition* 1990;10:357-382.
173. Gensler HL, Magdaleno M. Topical vitamin E inhibition of immunosuppression and tumorigenesis induced by ultraviolet irradiation. *Nat Cancer* 1991;15:97-106.
174. Farris MW, Pascoe GA, Reed DJ. Vitamin E reversal of the effect of extracellular calcium on chemically-induced toxicity in hepatocytes. *Science* 1985;227:751-754.
175. Glascock PA, Gifford E, Farber, JL. Effects of vitamin E on the killing of cultured hepatocytes by *tert*-butyl hydroperoxide. *Mol Pharmacol* 1992;41:1155-1162.
176. Carini R, Poli G, Dianzani MU, Maddipati SP, Slater TF, Cheeseman KH. Comparative evaluation of the antioxidant activity of  $\alpha$ -tocopherol,  $\alpha$ -tocopherol polyethylene glycol 1000 succinate and  $\alpha$ -tocopherol succinate in isolated hepatocytes and liver microsomal suspensions. *Biochem Pharmacol* 1990;39:1597-1601.
177. Farris MW. Oxygen toxicity: Unique cytoprotective properties of vitamin E succinate in hepatocytes. *Free Radical Biol Med* 1990;9:333-343.
178. Farris MW, Bryson KF, Gu XY, Smith JD, Walton LP. Superior protection against oxidative injury with hydrophilic tocopherol esters in rat hepatocytes. *Toxicologist* 1995;15:160(abstr.).
179. Harrison WH, Gander JE, Blakeley ER, Boyer PD. Interconversions of  $\alpha$ -tocopherol and its oxidation products. *Biochim Biophys Acta* 1986;21:150-158.
180. Oxman MA, Cohen LA. Reductive cyclization of  $\alpha$ -tocopherolquinone. *Biochim Biophys Acta* 1966;113:412-413.
181. Hayashi T, Kanetsoshi A, Nakamura M, Tamura M, Shirahama H. Reduction of  $\alpha$ -tocopherolquinone to  $\alpha$ -tocopherolhydroquinone in rat hepatocytes. *Biochem Pharmacol* 1992;44:489-493.
182. Kohar I, Baca M, Starna C, Stocker R, Southwell-Keely PT. Is  $\alpha$ -tocopherol a reservoir for  $\alpha$ -tocopherolhydroquinone? *Free Radical Biol Med* 1995;19:197-207.
183. Frei B. Reactive oxygen species and antioxidant vitamins: Mechanisms of action. *Am J Med* 1994;97:35S-13S.
184. Miller DM, Aust SD. Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation. *Arch Biochem Biophys* 1989;271:113-119.
185. Washko PW, Wang Y, Levine M. Ascorbic acid recycling in human neutrophils. *J Biol Chem* 1993;268:15531-15535.
186. Winkler BS, Orselli SM, Rex TS. The redox couple between glutathione and ascorbic acid: A chemical and physiological perspective. *Free Radical Biol Med* 1994;17:333-349.
187. Wells, WW, Xu, DP, Ynag, Y, Rocque, PA. Mammalian thioredoxinase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J Biol Chem* 1990;265:15361-15364.
188. Reed DJ. Interaction of vitamin E, ascorbic acid, and glutathione in protection against oxidative stress. In: Packer L, ed. *Vitamin E in Health and Disease*. New York: Marcel Dekker, 1992;269-281.

# **MOLECULAR METHODS FOR VIRUS DETECTION**

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## Detection Methods Using Chemiluminescence

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- I. General Introduction
- II. Chemiluminescence Methods
  - A. Dioxetanes
  - B. Luminol
  - C. Acridinium Esters
  - D. Electrochemiluminescence
  - E. Bioluminescence
- III. Instrumentation for Chemiluminescence Assays
- IV. Chemiluminescence Assays for Virus Detection
  - A. DNA Hybridization Assay Formats
  - B. Chemiluminescence Detection Systems
- V. Chemiluminescence Detection Protocols
  - A. Hepatitis B Virus
  - B. Herpes Simplex Virus
- VI. Conclusion
- References

### I. GENERAL INTRODUCTION

Chemiluminescence, the emission of light from a chemical reaction, has been studied extensively for many decades. Chemiluminescent processes constitute a very special class of chemical reactions in which products (or intermediates) are produced in electronically excited states that are very short-lived and rapidly decay with concomitant emission of light. Similar chemiluminescent reactions, called bioluminescence, occur in nature in species as diverse as the firefly (*Photinus pyralis*), marine bacteria (*Vibrio harveyi*), and others. Most chemiluminescence reactions involve oxidations of



a variety of organic compounds as well as naturally occurring materials, resulting in the generation of light-emitting excited states. This phenomenon was first described with synthetic organic compounds in 1877 (Radziszewski, 1877).

Chemiluminescent reactions do not produce very high intensity light signals because of many efficient quenching processes that compete with the radiative decay of the excited states. Nevertheless, chemiluminescence has been used effectively as a very sensitive detection system in many applications (Carter and Kricka, 1982; Harber, 1982; Kricka and Carter, 1982), largely because no background light signals are generated since the emitting excited state is created in a dark chemical reaction (compared to scattered excitation light in fluorescence.) Therefore, in theory, every photon detected is a true signal of the assay. This feature of chemiluminescent molecules—coupled with long shelf-life, elimination of hazards associated with the use of radioisotopes, and their detectability at  $10^{-21}$  moles (detection of alkaline phosphatase with chemiluminescent dioxetane substrate; Kricka, 1992)—makes them ideal as a reporter system for immunoassays and DNA probe hybridization assays.

In this chapter we describe the use of various chemiluminescence methodologies for the detection of viruses in DNA hybridization assays. A short discussion of instrumentation used in chemiluminescence measurement is also included.

## II. CHEMILUMINESCENCE METHODS

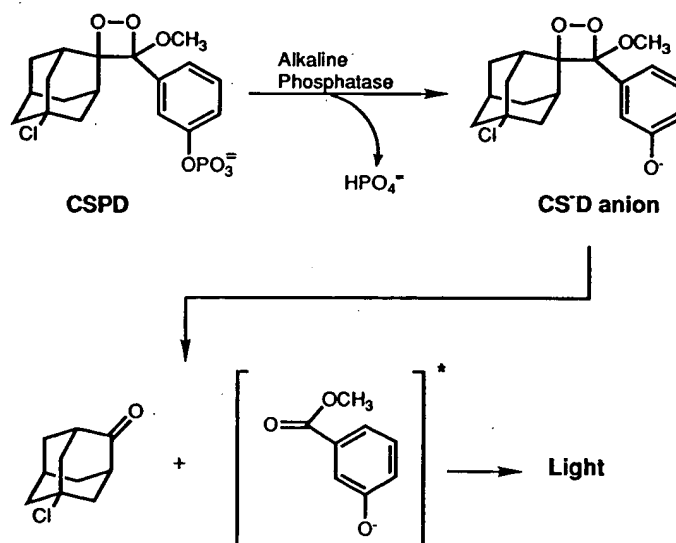
### A. Dioxetanes

Dioxetanes are four-membered cyclic peroxides that have been implicated as short-lived unstable intermediates in oxidation reactions that result in chemiluminescence (McCapra, 1966). Thus, 1,2-dioxetanes differ from most other chemiluminescence systems because these compounds do not require oxidation to emit light. Recently developed 1,2-dioxetanes that can be activated to luminesce by enzymes have been used successfully for bio-analyte detection. Dephosphorylation of adamantyl- and derivatized adamantyl-1,2-dioxetane phosphate substrates, such as AMPPD® [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-tricyclo[3.3.1.1<sup>3,7</sup>]decan}-4-yl)phenyl phosphate] and CSPD® [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan}-4-yl)phenyl phosphate] by alkaline phosphatase results in the formation of a destabilized anion that fragments further to form an excited state of methyl *meta*-oxybenzoate anion that emits light at

477 nm (Fig. 1; Bronstein *et al.*, 1989a,1991; Bronstein and Dimond, 1990; Bronstein and Sparks, 1992).

1,2-Dioxetane substrates for alkaline phosphatase are widely used in DNA hybridization assays (Bronstein, *et al.*, 1990; Pollard-Knight *et al.*, 1990b; Tumolo *et al.*, 1992). DNA probes are labeled with alkaline phosphatase either indirectly, with a biotin or hapten label followed by binding streptavidin- or antibody-alkaline phosphatase conjugates, or directly by covalent bonding to enzyme (oligonucleotide probes). Biotin has been the most popular ligand for indirect labeling, but hapten labels other than biotin have also been employed including digoxigenin, fluorescein, and 2,4-dinitrophenyl. Dioxetane-based chemiluminescent indirect labeling and detection systems for DNA hybridization assays, as well as for immunoassays and DNA sequencing, are widely available from many commercial suppliers. With a nick-translated biotinylated DNA probe, as little as 380 fg ( $7.9 \times 10^4$  copies) of target pBR322 DNA can be detected on a Southern blot (Bronstein *et al.*, 1990). Using digoxigenin-labeled random-primed DNA probes with a membrane-based assay and photographic film detection, a sensitivity level of 10–50 fg of target DNA was obtained for the detection of purified cytomegalovirus (CMV) or parvovirus B19 DNA (Musiani *et al.*, 1991a).

Direct labeling of oligonucleotide probes with alkaline phosphatase (Jablonski *et al.*, 1986) is possible with systems from Promega (Madison,



**Figure 1** Chemiluminescent decomposition of CSPD® 1,2-dioxetane triggered by enzymatic dephosphorylation.

WI) and Cambridge Research Biochemicals (Wilmington, DE). The detection of a single copy gene in 0.25  $\mu$ g human genomic DNA with Southern blot analysis has been achieved with an alkaline phosphatase-labeled oligonucleotide probe (Cate *et al.*, 1991).

Comparisons have shown that the sensitivity of alkaline phosphatase-dioxetane chemiluminescence detection is comparable to or better than that of  $^{32}$ P-based detection. In a human genomic Southern blot analysis of the tissue plasminogen activator gene, the sensitivity achieved with an alkaline phosphatase-labeled oligonucleotide probe was 12-fold higher than that achieved with the same  $^{32}$ P-5'-end-labeled probe, and the speed of detection was enhanced 40-fold with the alkaline phosphatase-labeled probe (Cate *et al.*, 1991). Slot blot hybridization of human serum samples with the alkaline phosphatase-labeled AmpliProbe® system (ImClone Systems, New York) showed a higher sensitivity in the detection of hepatitis B virus (HBV) relative to  $^{32}$ P-labeled nick-translated probes (Yang *et al.*, 1991). Similar sensitivities were obtained with an indirect digoxigenin-labeled probe and a random-primed  $^{32}$ P-labeled probe in a dot blot hybridization assay for amplified human immunodeficiency virus type 1 (HIV-1) DNA (Zachar *et al.*, 1991).

The sensitivity of alkaline phosphatase-dioxetane chemiluminescence detection has been shown to be superior to other nonisotopic systems based on colorimetric detection in membrane-based hybridization assays (Bronstein and Kricka, 1989; Bronstein and Voyta, 1989; Bronstein *et al.*, 1989c; Musiani *et al.*, 1991a, 1992). Finally, alkaline phosphatase-dioxetane detection has been demonstrated to be two to five times more sensitive than enhanced luminol chemiluminescent detection (described subsequently) in a solution hybridization assay system (Clyne *et al.*, 1989; Urdea *et al.*, 1990). Furthermore, the alkaline phosphatase-dioxetane detection system consists of fewer components, which are more stable than those required for an enhanced luminol chemiluminescent reaction (Beck and Köster, 1990). Although several other alkaline phosphatase-based chemiluminescent assays also exist, involving alternative substrates and coupled reactions, the most sensitive and widely used assays are those with 1,2-dioxetane substrates (for review of alternative systems, see Kricka, 1991).

## B. Luminol

Luminol and other cyclic diacylhydrazide derivatives can be oxidized in the presence of peroxide and peroxidase to generate an unstable intermediate in the excited state that chemiluminesces. Luminols can be used as direct chemiluminescent labels or as the chemiluminescent detectors of a peroxidase enzyme label (Kricka, 1991). Activation of luminol chemiluminescence with horseradish peroxidase (HRP) using an enhanced luminol system (en-

hanced chemiluminescence, ECL) has been done in DNA hybridization assays (Matthews *et al.*, 1985; Durrant *et al.*, 1990; Durrant, 1992) and immunoassays (reviewed by Bronstein and Sparks, 1992; Whitehead *et al.*, 1983; Thorpe *et al.*, 1985; Kricka *et al.*, 1987).

DNA probes can be labeled indirectly with HRP by binding streptavidin-HRP or anti-hapten-HRP conjugates or covalently by direct enzyme conjugation with oligonucleotides and longer double-stranded DNAs. The detection of single copy genes in 0.5  $\mu$ g human genomic DNA has been reported with indirectly labeled probes (Simmonds *et al.*, 1991). Direct HRP-labeled DNA probes have been used for both membrane-based DNA hybridization assays (Pollard-Knight *et al.*, 1990a; Simmonds *et al.*, 1991) and solution-phase hybridization assays (Urdea *et al.*, 1990). Detection of a single-copy gene on a Southern blot of <2  $\mu$ g human genomic DNA, with a sensitivity of <1 amol target DNA, has been demonstrated (Pollard-Knight *et al.*, 1990a) using direct HRP-labeled probes 0.3–5.1 kb in length. Similar sensitivity (1 amol target DNA) was also reported by Durrant *et al.* (1990). Both indirect and direct HRP labeling systems for nucleic acids and detection systems for HRP-catalyzed chemiluminescent reactions (ECL gene detection system) are available from Amersham (Arlington Heights, IL).

### C. Acridinium Esters

Acridinium esters (AE) are direct chemiluminescent labels for antibodies (Weeks *et al.*, 1983) and DNA probes (Septak, 1989; Nelson and Kacian, 1990; Nelson *et al.*, 1992), in contrast to dioxetane and luminol systems, in which an enzyme label catalyzes the chemiluminescent reaction. *N*-Methyl acridinium esters react with hydrogen peroxide under basic conditions to yield an excited state *N*-methylacridone which emits light at 430 nm (reviewed by Nelson and Kacian, 1990). Oligonucleotide DNA probes can be labeled covalently with AEs by reaction of modified *N*-hydroxysuccinimide-AE with a primary alkyl amine on a linker arm that was previously incorporated during oligonucleotide synthesis (Nelson and Kacian, 1990). Preparation of AE-labeled oligonucleotide probes has also been described by Septak (1989). The AE label does not affect probe hybridization characteristics; relatively large amounts of clinical specimen material may be used without interfering with hybridization and detection of AE-labeled probes.

Probe hybridization and detection reactions are performed in solution, using either separation or nonseparation formats. In a separation or heterogeneous assay, hybridized probe may be separated and detected by selective binding to microspheres, which can be separated from solution magnetically. In a nonseparation or homogeneous format, also termed a hybridization protection assay (HPA), the ester bond of the unhybridized probe can be hydrolyzed by differential chemical hydrolysis, thus rendering its AE label

nonchemiluminescent, whereas the AE label of the hybridized probe is minimally affected (Nelson and Kacian, 1990). This type of assay is possible because hybridization provides an intercalation site for the AE label, thereby protecting the AE molecule residing in the hybridized region from hydrolysis (Arnold *et al.*, 1989).

The sensitivity of this detection system is approximately  $5 \times 10^{-19}$  mol AE-labeled oligonucleotide, and the linear dynamic range is greater than four orders of magnitude (Nelson and Kacian, 1990). Similar sensitivities for the detection of an amplified *gag* sequence (4 HIV proviral copies per 150,000 cells) were achieved with colorimetric, chemiluminescence and  $^{32}\text{P}$ -labeling methods (Ou *et al.*, 1990; Rapier *et al.*, 1993). Schmidt (1991) was able to detect 0.05 fmol target HIV-1 DNA with AE-labeled *gag* probes and obtained greater sensitivity with chemiluminescence than with the same  $^{32}\text{P}$ -end-labeled probe in a dot blot hybridization assay.

#### D. Electrochemiluminescence

Electrochemiluminescence is a process in which the excited state products are generated via an electrochemical reaction (Faulkner and Glass, 1982). Electrochemiluminescence occurs when specific metal chelates such as ruthenium (II) tris(bipyridyl)  $[\text{Ru}(\text{bpy})_3^{2+}]$ , utilized as labels, undergo a series of chemical reactions at an electrode surface. Electrochemiluminescent labels for DNA hybridization assays have been utilized in a highly sensitive, simple, and versatile assay system. Oligonucleotide probes, synthesized with a free 5'-amino group, are readily labeled with  $\text{Ru}(\text{bpy})_3^{2+}$ -NHS ester (Blackburn *et al.*, 1991; Kenten *et al.*, 1991). Alternatively, oligonucleotide probes may be labeled during synthesis by incorporating labeled phosphoramidites (Kenten *et al.*, 1992; DiCesare *et al.*, 1993).

Electrochemiluminescent labels are relatively small molecules (~1000 dalton) that are extremely stable and may be coupled to nucleic acids, haptens, or proteins without affecting immunoreactivity or hybridization characteristics. The dynamic range for detection of these labels has been reported to be over six orders of magnitude (Blackburn *et al.*, 1991). These advantages, compared with other nonisotopic detection methods, provide potential wide utility in automated nonradioactive clinical diagnostic assays, including both DNA hybridization and immunoassay formats. A disadvantage of electrochemiluminescence, however, is a need for specialized instrumentation that can induce generation of electrochemically-excited states coupled with sensitive light detection.

Blackburn *et al.* (1991) used electrochemiluminescence detection with a DNA probe assay to quantify polymerase chain reaction (PCR)-amplified HIV-1 *gag* sequences. Double-stranded biotinylated PCR product was captured on streptavidin-coated microparticles and treated with alkali.

$\text{Ru}(\text{bpy})_3^{2+}$ -labeled oligonucleotide probe was then hybridized to the particle-bound DNA, washed, and quantified. A linear response was generated over the range of 50 to 2000 gene copies, and the detection of less than 10 copies of the HIV-1 *gag* was attained. An automated system for electrochemiluminescence quantification of PCR products (QPCR System 5000; Perkin-Elmer Corporation, Norwalk, CT) has been developed (DiCesare *et al.*, 1993) and is used for detection of viral disease. This system provides detection limits of 10–200 amol and a linear dynamic range greater than three orders of magnitude. The system has been used for the detection of HIV-1 over a range of 3 to  $10^6$  copies of target DNA (Wages *et al.*, 1993).

Because of the electrogeneration of the emitting species, which requires contact of the metal chelate label with an electrode, it is difficult to envision that simple membrane-based blotting assays that can be imaged on film could be designed using electrochemiluminescence.

### E. Bioluminescence

Bioluminescent reactions, a special class of chemiluminescent reactions that occur in nature and are catalyzed by a luciferase or photoproteins, offer an alternative method for luminescence detection of protein and DNA (Kricka, 1991). Two bioluminescent reaction systems have been used for DNA hybridization assays, both of which are coupled enzymatic reactions. One system, used for membrane-based DNA hybridization, couples the production of D-luciferin from D-luciferin-O-phosphate, catalyzed by alkaline phosphatase (as a direct or indirect label) and the oxidation of D-luciferin, catalyzed by firefly luciferase, with concomitant light emission (Hauber and Geiger, 1987, 1988; Hauber *et al.*, 1988, 1989; Geiger, 1992). The other system, used with both membrane-based and solution hybridization assays, couples reactions catalyzed by glucose-6-phosphate dehydrogenase (G6PDH), NAD(P)H: FMN oxidoreductase, and marine bacterial luciferase to produce the light (Balaguer *et al.*, 1989a,b, 1991a,b; Nicolas *et al.*, 1990, 1992). Although bioluminescence-based DNA detection systems have not become as widely used as chemiluminescence systems for DNA hybridization assays, they do offer another alternative for sensitive nonradioactive biomolecule detection.

## III. INSTRUMENTATION FOR CHEMILUMINESCENCE ASSAYS

A wide spectrum of instruments is currently available for recording and quantifying chemiluminescent signal intensities. These instruments, known as luminometers, use a light detector that consists of a photomultiplier tube

in photon counting mode, positioned close to the light source (microtiter plate or tube) to maximize photon collection efficiency. Among commercially available luminometers, semi-automated tube instruments such as the AutoClinilumat LB952T (Berthold/EG&G, Wallac, Inc., Gaithersburg, MD) and microtiter plate readers such as the ML 1000 (Dynatech Laboratories, Chantilly, VA) are most popular (reviewed by Bronstein and Kricka, 1990; Stanley, 1992a,b,1993b).

Chemiluminescence signals originating from blotting experiments performed on membranes can be detected by imaging on X-ray or instant photographic films. These films offer simple, convenient, and inexpensive detectors of chemiluminescence that can be used successfully for qualitative determinations and some signal quantification. Camera luminometers that house instant photographic film are suitable for the detection of light emission from blots and microtiter plate wells, and are available from Amersham, Analytical Luminescence Laboratory (San Diego, CA), Dynatech Laboratories, and Tropix, Inc. (Bedford, MA).

Finally, photon-counting cameras are available and are most suitable for the detection and accurate quantification of low-light signals. This instrumentation usually consists of a light detector such as a silicon target, silicon diode array, or charge-coupled device (CCD) coupled to a lens system, a controller, and a digital image processor. Since most of these camera systems are capable of imaging in two dimensions, micro- and macroscopic luminescent specimens can be analyzed spatially and temporally. The Argus-100/CL (Hamamatsu Corporation, Photonic Microscopy, Inc., Oak Brook, IL) is a photon-counting imaging device that has been used in the detection of blotted proteins (Hauber *et al.*, 1988). The Star I CCD cooled camera system (Photometrics Ltd., Tucson, AZ) exhibits very low dark current background and a wide dynamic range and has been used successfully to detect protein and nucleic acid analytes in solution and on membranes (Martin and Bronstein, 1993, 1994.).

#### IV. CHEMILUMINESCENCE ASSAYS FOR VIRUS DETECTION

The combination of DNA hybridization assays with chemiluminescence detection methods has enabled the development of rapid, sensitive, quantitative, nonradioactive assays that are amenable to automation. DNA hybridization technology is becoming accepted as a reliable clinical laboratory technique for the identification of infectious organisms and has fueled the need for more rapid, sensitive, and automated assay formats. Culture assay methods are laborious, time-consuming, and costly, and sometimes impossi-

ble to use. Antigen-based detection assays including fluorescent antibody and immunoassay techniques, although faster and automatable, are often less sensitive than culture techniques. With the advent of technologies such as PCR, DNA probe methods offer rapid, easy, and highly sensitive assay formats. DNA hybridization assays using radioactive labels are sensitive and are easily quantified, but health, environmental, disposal, and cost concerns render these systems less than ideal as widely used clinical assays.

Chemiluminescence methods for the detection of viral agents as well as other microorganisms have become widely used (Table 1), and continued development will certainly expand their applications in research and clinical diagnostic tests. More traditional immunoassays have also been developed and used with chemiluminescence for the detection of various viral antigens and the assessment of immune status with respect to viruses (selected references in Table 1). A survey of commercially available products that incorporate chemiluminescence or bioluminescence techniques and reagents for specific assays and nonspecific detection systems is available (Stanley, 1993a,b).

### **A. DNA Hybridization Assay Formats**

Several DNA hybridization assay formats including membrane-based, solution, and *in situ* hybridization have been coupled with chemiluminescence for the detection of viruses and other infectious agents. Membrane-based chemiluminescent hybridization assays have employed either 1,2-dioxetane substrates for alkaline phosphatase or the enhanced chemiluminescence reaction of luminol and HRP, and are imaged on X-ray or photographic films or imaged directly and quantified using a CCD camera system. Solution hybridization assays are performed with 1,2-dioxetanes, luminol, and AE labels, and the emitted light signal is measured in a luminometer. Electrochemiluminescent labels are also used for solution hybridization assays and are detected with an instrument combining an electrochemical flow cell, a potentiostat, and a photomultiplier tube. *In situ* hybridization has been performed using both 1,2-dioxetanes and enhanced luminol with either photographic film detection or a CCD camera system.

### **B. Chemiluminescence Detection Systems**

#### **1. Dioxetanes**

Alkaline phosphatase-dioxetane chemiluminescence systems have been used in a wide variety of DNA hybridization assays for detection of infectious



TABLE 1

Selected Studies That Have Used Chemiluminescence to Detect Viruses, *Chlamydia trachomatis*, and Other Microorganisms

Agent	Assay <sup>a</sup>	Reference
Barley yellow dwarf virus	MH	Fouly <i>et al.</i> (1992) (DX)
Bluetongue virus	PCR/H	Akita <i>et al.</i> (1993)
Bovine enteric coronavirus	MH	Collomb <i>et al.</i> (1992) (LU)
Bovine immunodeficiency-like virus	IA	Jacobs <i>et al.</i> (1992)
Bovine leukosis virus	IA	Miliukiene <i>et al.</i> (1991)
Bursal disease virus	H	Akin <i>et al.</i> (1993) (AP)
Chicken anemia virus	PCR/MH	Tham and Stanislawek (1992a,b) (DX)
Cytomegalovirus	MH	Musiani <i>et al.</i> (1991a,1992) (DX); Yang <i>et al.</i> (1991) (DX)
Dengue virus	PCR/H	Henchal <i>et al.</i> (1991) (DX)
Enterovirus (poliovirus)	MH	Fuchs <i>et al.</i> (1993) (DX)
Epstein-Barr virus	MH	Yang <i>et al.</i> (1991) (DX)
	PCR/MH	Vlieger <i>et al.</i> (1992) (LU)
	MH	Martinez and Weiss (1993) (AP)
Feline infectious peritonitis virus	IA	Pollini <i>et al.</i> (1993) (LU)
Grapevine closterovirus	MH	Bronstein <i>et al.</i> (1989c) (DX); Farmar and Castaneda (1991) (DX); Yang <i>et al.</i> (1991) (DX)
Hepatitis B virus	PCR/MH	Escarceller <i>et al.</i> (1992) (DX)
	SH	Urdea <i>et al.</i> (1987,1990) (LU; LU, DX)
	IA	Khalil <i>et al.</i> (1991a,b) (AE); Bouveresse and Bourgeois (1992) (AP); Boxall (1992) (LU); McCartney <i>et al.</i> (1993) (LU)
Hepatitis C virus	IM	Ireland and Samuel (1989) (LU); Robertson <i>et al.</i> (1991) (AE); Weare <i>et al.</i> (1991) (AE)
	PCR/H	Geiger and Caselmann (1992)
	IA	Khalil <i>et al.</i> (1991b) (AE)
Herpes simplex virus	MH	Bronstein and Voyta (1989) (DX)
	ISH	Bronstein and Voyta (1989) (DX)
	PCR/H	Puchhammerstoekl <i>et al.</i> (1993) (AP)
	IA	Pronovost <i>et al.</i> (1981) (LU); Dalessio and Ashley (1992) (LU)
Human immunodeficiency virus	PCR/MH	Conway <i>et al.</i> (1990) (AP); Zachar <i>et al.</i> (1991) (DX)
	PCR/SH	Ou <i>et al.</i> (1990) (AE); Blackburn <i>et al.</i> (1991) (EL); Schmidt (1991) (AE); Schmidt and Gschnait (1991); Gudibande <i>et al.</i> (1992) (EL); Kenten <i>et al.</i> (1992) (EL); Suzuki <i>et al.</i> (1992) (DX); Rapier <i>et al.</i> (1993) (AE); Wages <i>et al.</i> (1993) (EL)
	PCR	Bettens <i>et al.</i> (1991) (DX)
	BH	Ishii and Ghosh (1993) (AP)

Human papilloma virus	ISH	Bronstein <i>et al.</i> (1989b) (DX)
	RT	Suzuki <i>et al.</i> (1993) (DX)
	IA	Khalil <i>et al.</i> (1991b) (AE); Jacobs <i>et al.</i> (1992)
Human T-cell leukemia virus	PCR/SH	Balaguer <i>et al.</i> (1991b) (BL); Kenten <i>et al.</i> (1991) (EL)
	MH	Sarkar <i>et al.</i> (1993)
	ISH	Hawkins and Cumming (1990) (LU)
Human T-cell lymphotropic virus	IA	Khalil <i>et al.</i> (1991b) (AE)
	IM	Kuroda <i>et al.</i> (1992) (LU)
	IA	Papsidero <i>et al.</i> (1992) (LU)
Influenza virus	MH	McKimm-Breschkin (1992) (DX)
	IM	Arenkov <i>et al.</i> (1991)
Lentivirus	RT	Cook <i>et al.</i> (1992) (DX)
Parvovirus	MH	Musiani <i>et al.</i> (1991a,b) (DX)
	IA	O'Neill and Coyle (1992) (LU)
Potato virus Y, potato spindle tuber viroid	MH	Welnicki and Hiruki (1993) (AP)
Potato, pome fruit viroid	MH	Podleckis <i>et al.</i> (1993) (AP)
Respiratory syncytial virus, rotavirus	IM	Hornsleth <i>et al.</i> (1988) (LU)
Rubella virus	IM	Chanteloup <i>et al.</i> (1992) (AP)
Varicella zoster virus	PCR/H	Eis-Hubinger <i>et al.</i> (1992) (AP)
Bacteria	MH	Gustafsson and Persing (1992) (LU)
	?	Daly <i>et al.</i> (1991)
<i>Chlamydia trachomatis</i>	SH	Clyne <i>et al.</i> (1989) (DX); Urdea <i>et al.</i> (1989) (DX); Gratton <i>et al.</i> (1990) (AE); Mercer <i>et al.</i> (1990) (AE); Iwen <i>et al.</i> (1991) (AE)
	IA	Neman-Simha <i>et al.</i> (1991); Dumornay <i>et al.</i> (1992) (AE); Jang <i>et al.</i> (1992) (AE); Scieux <i>et al.</i> (1992a,b) (AE)
Mycobacteria	SH	Bull and Shanson (1992) (AE)
	SD/SH	Donahue <i>et al.</i> (1993) (DX)
<i>Neisseria gonorrhoeae</i>	PCR/MH	Sritharan and Barker (1991) (DX)
<i>Plasmodium falciparum</i>	SH	Urdea <i>et al.</i> (1989) (DX); Vlaspolter <i>et al.</i> (1993) (AE)
<i>Toxoplasma gondii</i>	PCR/MH	Barker <i>et al.</i> (1992) (DX)
	PCR/MH	Stauber <i>et al.</i> (1991) (LU)

<sup>a</sup> Assay formats include: BH, bead-based hybridization; H, hybridization; IA, immunoassay; IM, immunoblot; MH, membrane-based hybridization; PCR, polymerase chain reaction; RT, reverse transcriptase; SD, strand displacement; SH, solution hybridization.

<sup>b</sup> Chemiluminescent (CL) methods employed (if known): AE, acridinium ester; AP, alkaline phosphatase (most likely with dioxetane substrate); BL, bioluminescence; DX, dioxetane; EL, electrochemiluminescence; ILU, isoluminol; LU, enhanced luminol.

agents. Membrane-based hybridization assays have been used for the detection of HBV (Bronstein *et al.*, 1989c; Yang *et al.*, 1991; Escarceller *et al.*, 1992), herpes simplex virus (HSV-1) (Bronstein and Voyta, 1989), CMV (Musiani *et al.*, 1991a,1992; Yang *et al.*, 1991), HIV-1 (Zachar *et al.*, 1991), and other viral agents (Fouly *et al.*, 1992; Tham and Stanislawek, 1992a,b; Fuchs *et al.*, 1993). Solution hybridization assays include those for HBV (Urdea *et al.*, 1990), HIV-1 (Suzuki *et al.*, 1992), and *Chlamydia* (Clyne *et al.*, 1989; Urdea *et al.*, 1989). *In situ* hybridization assays have been performed with both HSV-1 infected cells (Bronstein and Voyta, 1989) and HIV-infected cells (Bronstein *et al.*, 1989b). Finally, assays for retroviruses based on the detection of reverse transcriptase activity can be coupled with chemiluminescence detection by measuring the enzymatic incorporation of digoxigenin-labeled nucleotides with anti-digoxigenin alkaline phosphatase and a dioxetane substrate (Suzuki *et al.*, 1993).

Commercially available detection systems incorporating dioxetanes include the AmpliProbe® system (ImClone Systems) for membrane-based hybridization assays for HBV, CMV, and EBV (Yang *et al.*, 1991), the Hybrid Capture™ System HBV DNA Assay (Murex Diagnostics Ltd., Kent, UK), and solution hybridization assay systems for *Chlamydia trachomatis* and HBV detection (Chiron Corporation; Clyne *et al.*, 1989; Urdea *et al.*, 1989,1990).

## 2. Luminol

DNA hybridization assays using the ECL system with direct HRP-labeled probes include detection of bovine enteric coronavirus in a slot blot hybridization assay (Collomb *et al.*, 1992) and a solution-phase hybridization assay for HBV DNA (Urdea *et al.*, 1987,1990). *In situ* hybridization for detection of human papillomavirus (HPV) type 16 has been performed with an indirect labeled probe (Hawkins and Cumming, 1990). ECL systems have also been used for the immunoassay detection of several viruses, including grapevine closterovirus (Pollini *et al.*, 1993) and parvovirus B 19 (O'Neill and Coyle, 1992).

## 3. Acridinium Esters

DNA hybridization assays incorporating AE-labeled probes have been developed for detection of several infectious agents from clinical samples, including *C. trachomatis*, *Neisseria gonorrhoeae*, fungal pathogens, mycobacteria, and several common bacterial pathogens (Nelson and Kacian, 1990). These assay systems, called PACE 2™ and ACCUPROBE™, are

available commercially through Gen-Probe, Inc. (San Diego, CA). The Gen-Probe system for screening for *Chlamydia* has been compared with both culture and nonculture antigen detection methods including enzyme immunoassays and immunofluorescent antibody tests (Gratton *et al.*, 1990; Mercer *et al.*, 1990; Iwen *et al.*, 1991). The PACE 2™ system can provide a rapid, reliable alternative to culture and immunoassay methods for the detection of *Chlamydia* from cervical samples (Iwen *et al.*, 1991). Solution hybridization (hybridization protection) assays with AE-labeled probes have been used for the detection of PCR-amplified HIV-1 DNA (Ou *et al.*, 1990; Schmidt, 1991; Rapier *et al.*, 1993).

In addition, AEs have also been used to label antibodies that have been incorporated into automated immunoassay formats for the detection of infectious agents and antibody screening from clinical samples (Khalil *et al.*, 1991a,b).

#### 4. Electrochemiluminescence

Electrochemiluminescence detection has been used in both manual (Blackburn *et al.*, 1991; Gudibande *et al.*, 1992; Kenten *et al.*, 1992) and automated (QPCR System 5000; Wages *et al.*, 1993) post-PCR amplification DNA hybridization assays for the detection of HIV-1 and HPV (Kenten *et al.*, 1991).

#### 5. Bioluminescence

Detection of asymmetric amplified papillomavirus sequences using solution-phase hybridization with a G6PDH-labeled oligonucleotide and solid-phase capture has been performed using a bioluminescence assay (Bala-guer *et al.*, 1991b).

### V. CHEMILUMINESCENCE DETECTION PROTOCOLS

#### A. Hepatitis B Virus

Hepatitis B "core antigen" DNA, immobilized on nylon membrane, is hybridized with an alkaline phosphatase-labeled oligonucleotide probe. Hybridized probe is then detected with the 1,2-dioxetane substrate AMPPD (Bronstein *et al.*, 1989c).

## 1. Materials

Hepatitis B core antigen plasmid DNA and alkaline phosphatase-labeled probe, included in a SNAP® Hybridization System, and GeneScreen Plus™ nylon membrane were obtained from NEN/DuPont (Boston, MA). AMPPD and CSPD are from Tropix.

## 2. Target DNA Preparation and Probe Hybridization

HBV "core antigen" (HBVc) plasmid DNA (100 ng;  $1.2 \times 10^{10}$  copies) was dissolved in 25  $\mu$ l sterile deionized H<sub>2</sub>O and serially diluted with 0.3 M NaOH to produce target DNA samples ranging in concentration from  $4.88 \times 10^3$  to  $0.98 \times 10^8$  copies/ $\mu$ l. Blots were prepared as described here:

1. Incubate diluted DNA samples at room temperature for 15 min to denature, and spot 1  $\mu$ l of each dilution onto dry membrane strips (1  $\times$  8 cm).
2. Rinse blots with 2 M NH<sub>4</sub>OAc and then with 0.6 M NaCl, 0.08 M sodium citrate, pH 7.0.
3. Prehybridize with 3 ml hybridization buffer [0.75 M NaCl, 0.075 M sodium citrate (5X SSC), 0.5% bovine serum, 0.5% polyvinylpyrrolidone, 1% sodium dodecyl sulfate (SDS), pH 7.0] for 15 min at 55°C.
4. Hybridize with hybridization buffer containing 1.0 nM alkaline phosphatase-labeled oligonucleotide probe for 30 min at 55°C.
5. Wash sequentially for 5 min each in:

1X SSC, pH 7.0, 1% SDS at room temperature

1X SSC, pH 7.0, 1% Triton X-100 at 55°C

1X SSC, pH 7.0, at room temperature

## 3. Chemiluminescence Detection

1. Wash hybridized blots with 0.1% bovine serum albumin (BSA), 0.05 M sodium carbonate, pH 9.5.
2. Saturate blot with 100  $\mu$ l 1.6 mM AMPPD in 0.1% BSA, 0.05 M sodium carbonate, 1.0 mM MgCl<sub>2</sub>, pH 9.5.

NOTE: Alternatively, an improved buffer (0.1 M diethanolamine, 1.0 mM MgCl<sub>2</sub>, pH 10.0) can be substituted for this wash, using 0.25 mM AMPPD or CSPD in this buffer for substrate incubation.

3. Place blots in a plastic pouch and image light emission in a camera

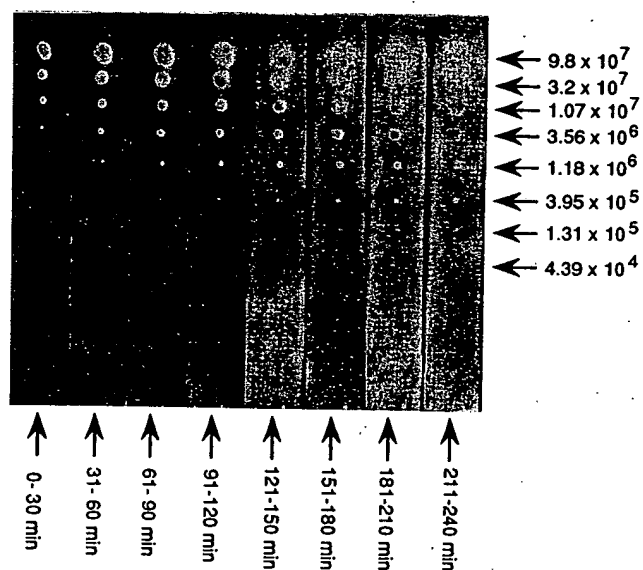
luminometer with Polaroid Instant Black and White Type 612 (ASA 20,000) photographic film.

NOTE: Alternatively, blots can be imaged on standard X-ray film.

4. Digitize photographic film image using a black and white RBP Reflectance Densitometer (Tobias Associates, Inc., Ivyland, PA).

#### 4. Results

Figure 2 shows a time course of the chemiluminescent DNA hybridization assay for HBVc antigen DNA. Serial dilutions of plasmid DNA were hybridized with alkaline phosphatase-labeled oligonucleotide probe, incubated with chemiluminescent substrate, and imaged on photographic film. Each photograph corresponds to a 30-min exposure. With this chemiluminescence assay,  $1.18 \times 10^6$  copies of HBVc DNA can be detected within 30 min of substrate incubation. After a 2-hr incubation,  $4.39 \times 10^4$  copies can be detected. In contrast, with the colorimetric bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate system,  $9.8 \times 10^7$  and  $1.07 \times 10^7$  copies can be detected after 30 min or 2 hr of substrate incubation,

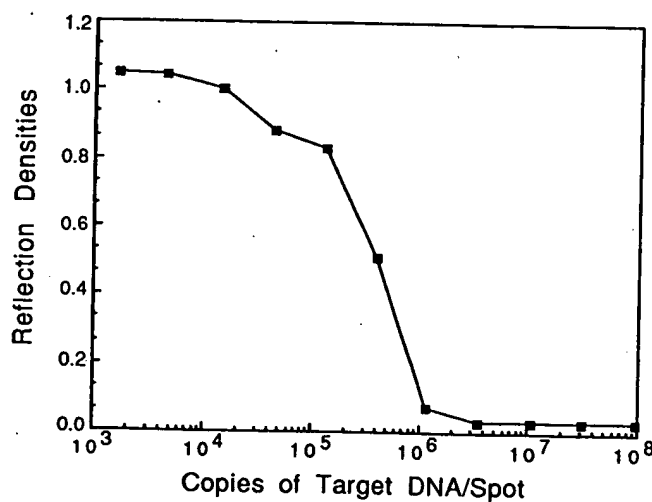


**Figure 2** Chemiluminescent detection of hepatitis B "core antigen" plasmid DNA with AMPPD substrate in alkaline phosphatase-based DNA hybridization assay. Reprinted with permission from Bronstein *et al.* (1989c).

respectively (results not shown). Quantitative results were obtained by measuring reflection densities from the imaged photographic film using a black and white reflection densitometer (Fig. 3). These values could be used to establish a dose-response curve for the reflection densities as a function of HBVc plasmid concentration, from which HBVc DNA levels in clinical specimens could be determined. Use of the improved chemiluminescence detection protocol, incorporating the diethanolamine substrate buffer and CSPD chemiluminescent substrate, results in even greater sensitivity for DNA hybridization assays and would increase the sensitivity of this HBV DNA assay. Imaging and quantification of this membrane-based assay with rapidly evolving CCD camera systems will likely provide even greater sensitivity and a greater linear dynamic range than that achieved with densitometry.

### 5. Summary

Chemiluminescent detection of HBV DNA has also been performed with the AmpliProbe® system (ImClone Systems). This signal amplification probe system incorporates multiple target-specific primary and multiple secondary probes, alkaline phosphatase-labeled oligonucleotides that hybridize to the primary probes, in a two-step hybridization system (Yang *et al.*, 1991). Chemiluminescence detection is performed with a dioxetane substrate. With



**Figure 3** Hepatitis B virus "core antigen" plasmid DNA hybridization assay. Reflection density vs. number of copies of target DNA. Densitometric analysis of the Polaroid instant black and white photographic film image [0.00 (white)–2.00 (black)]. Reprinted with permission from Bronstein *et al.* (1989c).

this system, 0.4 pg ( $1 \times 10^5$  copies) purified target HBV genomic DNA can be detected in a chemiluminescent slot blot assay (Farmer and Castaneda, 1991; Yang *et al.*, 1991). Identical assays performed with serum samples (25  $\mu$ l) demonstrated that this chemiluminescent DNA hybridization system has the same specificity and sensitivity as immunoassays and is more sensitive than a  $^{32}$ P-labeled nick-translated probe (Yang *et al.*, 1991).

Escarceller *et al.* (1992) report the use of digoxigenin-labeled probes, anti-digoxigenin alkaline phosphatase, and AMPPD for the direct detection of HBV sequences in human serum samples. These investigators achieved a limit of sensitivity of 2–5 pg, which was equivalent to that obtained with both colorimetric detection and a  $^{32}$ P-labeled probe. These researchers also used digoxigenin-labeled oligonucleotide primers for PCR amplification of HBV DNA purified from human serum, followed by immunological detection of the digoxigenin label (as described), a method that can be used in conjunction with alternatively labeled primers for multiple amplifications.

A chemiluminescent assay incorporating a solution-phase hybridization of synthetic oligonucleotides to target DNA, followed by solid-phase capture, labeling, and detection with either HRP or alkaline phosphatase-labeled oligonucleotides and chemiluminescent substrates has been used to achieve the detection of 0.2 pg ( $6 \times 10^4$  copies) HBV DNA in human serum samples in 4 hr. This solution DNA hybridization method includes novel labeling and amplification schemes and has been performed with both polystyrene bead and microtiter well capture systems (Urdea *et al.*, 1987, 1990).

Chemiluminescence techniques have also been used in the development of automated enzyme immunoassay systems for the detection of HBV in human sera (Khalil *et al.*, 1991a,b; Bouveresse and Bourgeois, 1992).

## B. Herpes Simplex Virus

Two chemiluminescent DNA hybridization assays for HSV, dot blot hybridization and *in situ* hybridization, are described here as originally reported by Bronstein and Voyta (1989). In these assays, HSV-1 plasmid DNA, immobilized on nylon membrane, or HSV-1-infected Vero cells, fixed and mounted on microscope slides, were hybridized with an alkaline phosphatase-labeled HSV-1 oligonucleotide probe and detected with AMPPD.

### 1. Materials

HSV-1 plasmid DNA and alkaline phosphatase-labeled oligonucleotide probe, included in a SNAP® Hybridization System, and GeneScreen Plus nylon membrane were obtained from NEN/DuPont. HSV-1-infected Vero



cells were provided by Drs. J. Kershner and E. Jablonski (Molecular Biosystems, San Diego, CA). AMPPD and Emerald™ luminescence-amplifying material are from Tropix.

## 2. Dot Blot Hybridization and Chemiluminescence Detection

This membrane hybridization protocol is similar to that described for HBV detection.

1. Serially dilute HSV-1 plasmid DNA in 0.3 M NaOH, denature, and spot 1- $\mu$ l aliquots onto dry membrane strips.
2. Prehybridize blots with hybridization buffer (0.5% BSA, 0.5% polyvinylpyrrolidone, 1% SDS) for 15 min at 55°C.
3. Hybridize with hybridization solution (containing alkaline phosphatase-labeled HSV-1 oligonucleotide probe) for 30 min at 55°C.
4. Wash sequentially for 5 min each in:

2X SSC, 1% SDS at room temperature

1X SSC, 1% Triton X-100 at 55°C

1X SSC, 1% Triton X-100 at room temperature

1X SSC at room temperature

5. Wash hybridized blots with 0.05 M sodium carbonate/bicarbonate, 1 mM MgCl<sub>2</sub>, pH 9.5 (substrate buffer).
6. Saturate blot with 1.6 mM AMPPD (in substrate buffer) for 5 min.

NOTE: As described for HBV detection, the diethanolamine buffer and 0.25 mM AMPPD or CSPD can be substituted in Steps 5 and 6 for increased sensitivity.

7. Image blots with Polaroid Type 612 Instant Black and White film.

NOTE: Alternatively, blots can be imaged on X-ray film.

## 3. In Situ Hybridization and Chemiluminescence Detection

1. Infect Vero cells with HSV-1 (MacIntyre strain) for 1 hr at room temperature.
2. Harvest cells with trypsin/versene after the addition of 2% fetal calf serum at 0, 2, 4, 6, 8, 10, 12, 24, and 48 hr.
3. Pellet cells, fix in 95% ethanol, and mount on glass microscope slides.

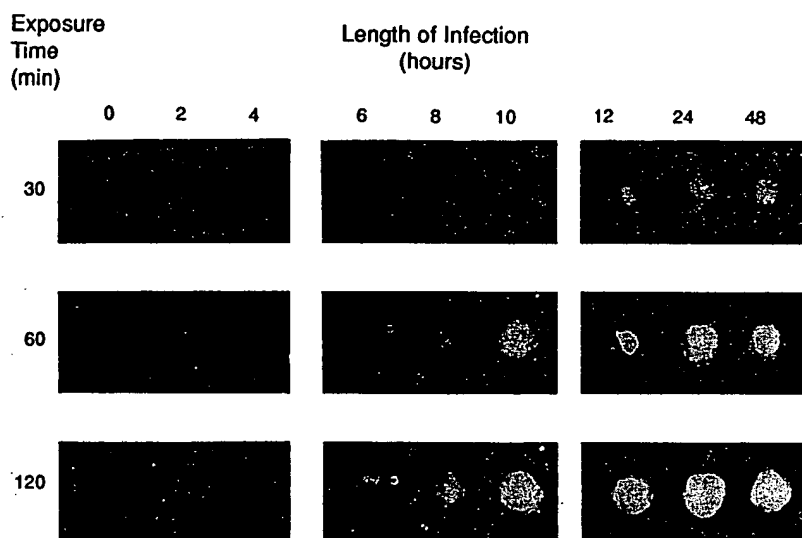
4. Treat mounted slides with 0.2 M HCl for 2 min, rinse with deionized water, and immerse in 70% ethanol. Prior to hybridization, remove slides from ethanol and dry.
5. Immerse slides in 0.1% BSA, 5X SSC for 15 min at 70°C. Treat with 0.3 M NaOH for 1 min at room temperature. Rinse with phosphate-buffered saline (PBS).
6. Hybridize cells with the alkaline phosphatase-labeled HSV-1 oligonucleotide probe at a concentration of 5 nM in 0.1% BSA, 5X SSC for 20 min at 60°C.
7. Wash slides briefly in hybridization buffer at 60°C, and then extensively with 1X SSC at 50°C.
8. Wash with 0.05 M sodium carbonate/bicarbonate, 1 mM MgCl<sub>2</sub>, pH 9.5 (substrate buffer).
9. Incubate with 0.8 mM AMPPD, 10% Emerald in substrate buffer for 5 min.
10. Place slides in a camera luminometer and expose to Polaroid Type 612 Instant Black and White film.

#### 4. Results

With the dot blot hybridization assay for HSV-1 plasmid DNA, detection limits achieved with the chemiluminescent substrate AMPPD are  $1.3 \times 10^5$  and  $1.4 \times 10^4$  copies of target HSV-1 DNA, with a 30-min exposure performed 1 hr after substrate addition and a 45-min exposure performed 4 hr after substrate addition, respectively (results not shown). The sensitivity achieved with AMPPD is 25- to 100-fold higher than that obtained with the colorimetric BCIP/NBT substrate system (results not shown). Fig. 4 shows the time course of viral infection assayed by *in situ* DNA hybridization with chemiluminescence detection. Use of the AMPPD chemiluminescent substrate enables the detection of HSV-1-infected cells within 6 hr postinfection. Again, with this assay format, CCD detection and imaging may provide even greater sensitivity than that achieved with photographic film.

#### 5. Summary

*In situ* hybridization with chemiluminescence detection has also been used to detect HIV-infected cells (Bronstein *et al.*, 1989b) and HPV type 16 in a cervical carcinoma cell line (Hawkins and Cumming, 1990). The latter protocol involved the use of biotinylated HPV 16 DNA probes (Enzo Diagnostics, New York), a streptavidin-HRP conjugate, and ECL detection re-



**Figure 4** Chemiluminescent detection of *in situ* DNA hybridization of herpes simplex virus I-infected Vero cells: time course of infection. Reprinted from Bronstein and Voyta, *Clinical Chemistry* (1989), 35, 1856–1857, Courtesy of the American Association for Clinical Chemistry, Inc.

agents coupled with a CCD imaging system. Detection of fewer than 10 HPV-positive cells (containing 600 copies of HPV 16 DNA per cell) among 10,000 HPV-negative cells on a single slide was achieved. However, this detection level is not necessarily the limit of sensitivity; with improved optical instrumentation, *in situ* hybridization coupled with CCD detection may provide a valuable diagnostic tool for the rapid and automated identification of viral sequences within cells.

## VI. CONCLUSION

Chemiluminescence detection technologies combined with DNA hybridization methods provide rapid, sensitive, nonradioactive, automatable assay formats for the clinical diagnosis of infectious agents, as well as for research use. Rapidly evolving chemiluminescent enzyme substrates and labels, techniques, and assay and detection instrumentation, coupled with continued advances in DNA hybridization technologies, will further refine and improve the specificity and sensitivity of chemiluminescent DNA detection methods, bringing them into more widespread use.

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## REFERENCES

- Akin, A., Wu, C. C., Lin, T. L., and Keirs, R. W. (1993). Chemiluminescent detection of infectious bursal disease virus with a PCR-generated nonradiolabeled probe. *J. Vet. Diagn. Invest.* 5, 166-173.
- Akita, G. Y., Glenn, J., Castro, A. E., and Osburn, B. I. (1993). Detection of bluetongue virus in clinical samples by polymerase chain reaction. *J. Vet. Diagn. Invest.* 5, 154-158.
- Arenkov, P. I., Berezin, V. A., and Starodub, N. F. (1991). Chemiluminescence fiber optic immunosensor for detecting antibodies to the influenza virus. *Ukr. Biokhim.* 63, 99-103.
- Arnold, L. J., Hammond, P. W., Wiese, W. A., and Nelson, N. C. (1989). Assay formats involving acridinium-ester-labeled DNA probes. *Clin. Chem.* 35, 1588-1594.
- Balaguer, P. T., Térouanne, B., Boussioux, A.-M., and Nicolas, J.-C. (1989a). Use of bioluminescence in nucleic acid hybridization reactions. *J. Biolumin. Chemilumin.* 4, 302-309.
- Balaguer, P., Térouanne, B., Eliaou, J. F., Humbert, M., Boussioux, A. M., and Nicolas, J. C. (1989b). Use of glucose-6-phosphate dehydrogenase as a new label for nucleic acid hybridization reactions. *Anal. Biochem.* 180, 50-54.
- Balaguer, P., Térouanne, B., Boussioux, A., and Nicolas, J. (1991a). Quantification of DNA sequences obtained by polymerase chain reaction using a bioluminescent adsorbent. *Anal. Biochem.* 195, 105-110.
- Balaguer, P., Térouanne, B., Boussioux, A. M., and Nicolas, J. C. (1991b). Papillomavirus quantification using asymmetric amplification and a rapid bioluminescent assay. In "Bioluminescence & Chemiluminescence: Current Status" (P. E. Stanley and L. J. Kricka, eds.), pp. 143-146. John Wiley & Sons, Chichester.
- Barker, R. H., Jr., Banchongaksorn, T., Courval, J. M., Suwonkerd, W., Rimwungtragoon, K., and Wirth, D. F. (1992). A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reactions. *Am. J. Trop. Med. Hyg.* 46, 416-426.
- Beck, S., and Köster, H. (1990). Applications of dioxetane chemiluminescent probes to molecular biology. *Anal. Chem.* 62, 2258-2270.
- Bettens, F., Pichler, W. J., and de Weck, A. L. (1991). Incorporation of biotinylated nucleotides for the quantification of PCR-amplified HIV-1 DNA by chemiluminescence. *Eur. J. Clin. Chem. Clin. Biochem.* 29, 685-688.
- Blackburn, G. F., Shah, H. P., Kenten, J. H., Leland, J., Kamin, R. A., Link, J., Peterman, J., Powell, M. J., Shah, A., Talley, D. B., Tyagi, S. K., Wilkins, E., Wu, T.-G., and Massey, R. J. (1991). Electrochemiluminescence detection for development of immunoassays and DNA probe assays for clinical diagnostics. *Clin. Chem.* 37, 1534-1539.
- Bouveresse, E., and Bourgeois, J. P. J. (1992). Chemiluminescent enzyme immunoassay for the detection of hepatitis B surface antigen (HBsAg). *Clin. Chem.* 38, 1090.
- Boxall, E. H. (1992). Enhanced luminescent assays for hepatitis markers: Assessment of post vaccine responses. *Arch. Virol. Suppl.* 4, 156-159.
- Bronstein, I., and Dimond, P. (1990). Chemiluminescent compounds for diagnostic tests. *Diagn. Clin. Test.* 28, 36-39.

- Bronstein, I., and Kricka, L. J. (1989). Clinical applications of luminescent assays for enzymes and enzyme labels. *J. Clin. Lab. Anal.* 3, 316-322.
- Bronstein, I., and Kricka, L. J. (1990). Instrumentation for luminescent assays. *Am. Clin. Lab.* 9(1), 33-37.
- Bronstein, I., and Sparks, A. (1992). Sensitive enzyme immunoassays with chemiluminescent detection. In "Immunochemical Assays and Biosensor Technology for the 1990s" (R. M. Nakamura, Y. Kasahara, and G. A. Rechnitz, eds.), pp. 229-250. American Society for Microbiology, Washington, D.C.
- Bronstein, I., and Voyta, J. C. (1989). Chemiluminescent detection of herpes simplex virus I DNA in blot and in-situ hybridization assays. *Clin. Chem.* 35, 1856-1857.
- Bronstein, I., Edwards, B., and Voyta, J. C. (1989a). 1,2-Dioxetanes: Novel chemiluminescent enzyme substrates. Applications to immunoassays. *J. Biolumin. Chemilumin.* 4, 99-111.
- Bronstein, I., Kerschner, J. H., Voyta, J. C., and Jablonski, E. G. (1989b). Chemiluminescent detection of HIV infected cells using in-situ hybridization assay technique. Presented at the V International Conference on AIDS, Montreal, Quebec, Canada, June 4-9.
- Bronstein, I., Voyta, J. C., and Edwards B. (1989c). A comparison of chemiluminescent and colorimetric substrates in a hepatitis B virus DNA hybridization assay. *Anal. Biochem.* 180, 95-98.
- Bronstein, I., Voyta, J. C., Lazzari, K. G., Murphy, O., Edwards, B., and Kricka, L. J. (1990). Rapid and sensitive detection of DNA in Southern blots with chemiluminescence. *Bio Techniques* 8, 310-313.
- Bronstein, I., Juo, R.-R., Voyta, J. C., and Edwards, B. (1991). Novel chemiluminescent adamantyl 1,2-dioxetane enzyme substrates. In "Bioluminescence and Chemiluminescence: Current Status" (P. Stanley and L. J. Kricka, eds.), pp. 73-82. John Wiley, Chichester.
- Bull, T., and Shanson, D. (1992). Evaluation of a commercial chemiluminescent gene probe system "AccuProbe" for the rapid differentiation of mycobacteria, including "MAIC X," isolated from blood and other sites, from patients with AIDS. *J. Hosp. Infect.* 21, 143-149.
- Carter, T. J. N., and Kricka, L. J. (1982). Analytical applications of chemiluminescence. In "Clinical and Biochemical Luminescence" (L. J. Kricka and T. J. N. Carter, eds.), pp. 135-151. Marcel Dekker, New York.
- Cate, R. L., Ehrenfels, C. W., Wusk, M., Tizard, R., Voyta, J. C., Murphy, O. J., and Bronstein, I. (1991). Genomic Southern analysis with alkaline-phosphatase-conjugated oligonucleotide probes and the chemiluminescent substrate AMPPD. *Genetic Analysis, Techniques Applications* 8, 102-106.
- Chanteloup, E., Clement, A., and Payne, J. (1992). Chemiluminescent enzyme immunoassay for the evaluation of the immune status to rubella virus. *Clin. Chem.* 38, 1085.
- Clyne, J. M., Running, J. A., Stempien, M., Stephens, R. S., Akhavan-Tafti, H., Schaap, A. P., and Urdea, M. S. (1989). A rapid chemiluminescent DNA hybridization assay for the detection of *Chlamydia trachomatis*. 4, 357-366.
- Collomb, J., Finance, C., Alabouch, S., and Laporte, J. (1992). Radioactive and enzymatic cloned cDNA probes for bovine enteric coronavirus detection by molecular hybridization. *Arch. Virol.* 125, 25-37.
- Conway, B., Adler, K. E., Bechtel, L. J., Kaplan, J. C., and Hirsch, M. S. (1990). Detection of HIV-1 DNA in crude cell lysates of peripheral blood mononuclear cells by the polymerase chain reaction and nonradioactive oligonucleotide probes. *J. Acq. Immune Def. Syndr.* 3, 1059-1064.
- Cook, R. F., Cook, S. J., and Issel, C. J. (1992). A nonradioactive micro-assay for released reverse transcriptase activity of a lentivirus. *Bio Techniques* 13, 380-386.
- Dalessio, J., and Ashley, R. (1992). Highly sensitive enhanced chemiluminescence immunodetection methods for herpes simplex virus type-2 Western immunoblot. *J. Clin. Microbiol.* 30, 1005-1010.

- Daly, J., Clifton, N., Seshkin, K., and Gooch, W. (1991). Use of rapid, nonradioactive DNA probes in culture confirmation tests to detect *Streptococcus agalactiae*, *Haemophilus influenzae*, and *Enterococcus* spp. from pediatric patients with significant infections. *J. Clin. Microbiol.* 29, 80-82.
- DiCesare, J., Grossman, B., Katz, E., Picozza, Ragusa, R., and Woudenberg, T. (1993). A high-sensitivity electrochemiluminescence-based detection system for automated PCR product quantification. *Bio Techniques*. 15, 152-157.
- Donahue, C., Jurgensen, S., Nycz, C., Schram, J. L., Shank, D., Vonk, G. P., and Walker, G. T. (1993). Detection of mycobacterial DNA using strand displacement amplification and a chemiluminescent microwell assay. *J. Biolumin. Chemilumin.* 8, 77.
- Dumornay, W., Roblin, P., Gelling, M., Hammerschlag, M., and Worku, M. (1992). Comparison of a chemiluminometric immunoassay with culture for diagnosis of chlamydial infections in infants. *J. Clin. Microbiol.* 30, 1867-1869.
- Durrant, I. (1992). Detection of horseradish peroxidase by enhanced chemiluminescence. In "Nonisotopic DNA Probe Techniques" (L. J. Kricka, ed.), pp. 167-183. Academic Press, San Diego.
- Durrant, I., Benge, L. C. A., Sturrock, C., Devenish, A. T., Howe, R., Roe, S., Moore, M., Scozzafava, G., Proudfoot, L. M. F., Richardson, T. C., and McFarthing, K. G. (1990). The application of enhanced chemiluminescence to membrane-based nucleic acid detection. *Bio Techniques* 8, 564-570.
- Eis-Hubinger, A. M., Kaiser, R., Kleim, J. P., Dlugosch, D., Estor, A., Kleeman, E., Lange, C. E., and Schneewis, K. E. (1992). Detection of varicella zoster virus infections using polymerase chain reaction. *Hautarzt* 43, 767-771.
- Escarceller, M., Rodriguez-Frias, F., Jardi, R., San-Segundo, B., and Eritja, R. (1992). Detection of hepatitis B virus DNA in human serum samples: Use of digoxigenin-labeled oligonucleotides as modified primers for the polymerase chain reaction. *Anal. Biochem.* 206, 36-42.
- Farmer, J. G., and Castaneda, M. (1991). An improved preparation and purification of oligonucleotide-alkaline phosphatase conjugates. *Bio Techniques* 11, 588-589.
- Faulkner, L. R., and Glass, R. S. (1982). Electrochemiluminescence. In "Chemical and Biological Generation of Excited States" (W. Adam, and G. Cilento, eds.), pp. 191-227. Academic Press, New York.
- Fouly, H. M., Domier, L. L., and D'Arcy, C. J. (1992). A rapid chemiluminescent detection method for barley yellow dwarf virus. *J. Virol. Meth.* 39, 291-298.
- Fuchs, F., Lepar, I., Kopecka, H., Garin, D., and Aymard, M. (1993). Use of cRNA digoxigenin-labelled probes for detection of enteroviruses in humans and in the environment. *J. Virol. Meth.* 42, 217-226.
- Geiger, C. P., and Caselmann, W. H. (1992). Non-radioactive hybridization with hepatitis C virus-specific probes created during polymerase chain reaction: A fast and simple procedure to verify hepatitis C virus infection. *J. Hepatol.* 15, 387-390.
- Geiger, R. E. (1992). Detection of alkaline phosphatase by bioluminescence. In "Nonisotopic DNA Probe Techniques" (L. J. Kricka, ed.), pp. 113-126. Academic Press, San Diego.
- Gratton, C. A., Lim-Fong, R., Prasad, E., and Kibsey, P. C. (1990). Comparison of a DNA probe with culture for detecting *Chlamydia trachomatis* directly from genital specimens. *Mol. Cell. Probes* 4, 25-31.
- Gudibande, S. R., Kenten, J. H., Link, J., Friedman, K., and Massey, R. J. (1992). Rapid non-separation electrochemiluminescent DNA hybridization assays for PCR products, using 3'-labelled oligonucleotide probes. *Mol. Cell. Probes* 6, 495-503.
- Gustaferro, C. A., and Persing, D. H. (1992). Chemiluminescent universal probe for bacterial ribotyping. *J. Clin. Microbiol.* 30, 1039-1041.
- Harber, M. J. (1982). Applications of luminescence in medical microbiology and hematology.

- In "Clinical and Biochemical Luminescence" (L. J. Kricka and T. J. N. Carter, eds.), pp. 189-218. Marcel Dekker, New York.
- Hauber, R., and Geiger, R. (1987). A new, very sensitive, bioluminescence-enhanced detection system for protein blotting. Ultrasensitive detection systems for protein blotting and DNA hybridization, I. *J. Clin. Chem. Clin. Biochem.* 25, 511-514.
- Hauber, R., and Geiger, R. (1988). A sensitive, bioluminescence-enhanced detection method for DNA dot-hybridization. *Nucleic Acid Res.* 16, 1213.
- Hauber, R., Miska, W., Schleinkofer, L., and Geiger, R. (1988). The application of a photon-counting camera in very sensitive, bioluminescence-enhanced detection systems for protein blotting. Ultrasensitive detection systems for protein blotting and DNA hybridization, II. *J. Clin. Chem. Clin. Biochem.* 26, 147-148.
- Hauber, R., Miska, W., Schleinkofer, L., and Geiger, R. (1989). New, sensitive, radioactive-free bioluminescence-enhanced detection system in protein blotting and nucleic acid hybridization. *J. Biolumin. Chemilumin.* 4, 367-372.
- Hawkins, E., and Cumming, R. (1990). Enhanced chemiluminescence for tissue antigen and cellular viral DNA detection. *J. Histochem. Cytochem.* 38, 415-419.
- Henchal, E. A., Polo, S. L., Vorndam, V., Yaemsiri, C., Innis, B. L., and Hoke, C. H. (1991). Sensitivity and specificity of a universal primer set for the rapid diagnosis of dengue virus infections by polymerase chain reaction and nucleic acid hybridization. *Am. J. Trop. Med. Hyg.* 45, 418-428.
- Hornsleth, A., Aaen, K., and Gundestrup, M. (1988). Detection of respiratory syncytial virus and rotavirus by enhanced chemiluminescence enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 26, 630-635.
- Ireland, D., and Samuel, D. (1989). Enhanced chemiluminescence ELISA for the detection of antibody to hepatitis B virus surface antigen. *J. Biolumin. Chemilumin.* 4, 159-163.
- Ishii, J. K., and Ghosh, S. S. (1993). Bead-based sandwich hybridization characteristics of oligonucleotide-alkaline phosphatase conjugates and their potential for quantitating target RNA sequences. *Bioconjugate Chem.* 4, 34-41.
- Iwen, P. C., Blair, T. M., and Woods, G. L. (1991). Comparison of the Gen-Probe PACE 2 system, direct fluorescent-antibody, and cell culture for detecting *Chlamydia trachomatis* in cervical specimens. *Am. J. Clin. Pathol.* 95, 578-582.
- Jablonski, E., Moomaw, E. W., Tullis, R. H., and Ruth, J. L. (1986). Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. *Nucleic Acids Res.* 14, 6115-6128.
- Jacobs, R. M., Smith, H. E., Gregory, B., Valli, V. E., and Whetstone, C. A. (1992). Detection of multiple retroviral infections in cattle and cross-reactivity of bovine immunodeficiency-like virus and human immunodeficiency virus type 1 proteins using bovine and human sera in a western blot assay. *Can. J. Vet. Res.* 56, 353-359.
- Jang, D., Sellors, J. W., Mahony, J. B., Pickard, L., and Chernesky, M. A. (1992). Effects of broadening the gold standard on the performance of a chemiluminometric immunoassay to detect *Chlamydia trachomatis* antigens in centrifuged first void urine and urethral swab samples from men. *Sex. Trans. Dis.* 19, 315-319.
- Kenten, J. H., Casadei, J., Link, J., Lupolid, S., Willey, J., Powell, M., Rees, A., and Massey, R. (1991). Rapid electrochemiluminescence assays of polymerase chain reaction products. *Clin. Chem.* 37, 1626-1632.
- Kenten, J. H., Gudibande, S., Link, J., Willey, J. J., Curfman, B., Major, E. O., and Massey, R. J. (1992). Improved electrochemiluminescent label for DNA probe assays: Rapid quantitative assays of HIV-1 polymerase chain reaction products. *Clin. Chem.* 38, 873-879.
- Khalil, O. S., Hanna, C. F., Huff, D., Zurek, T. F., Murphy, B., Pepe, C., and Genger, K. (1991a). Reaction tray and noncontract transfer method for heterogeneous chemiluminescence immunoassays. *Clin. Chem.* 37, 1612-1617.

- Khalil, O. S., Zurek, T. F., Tryba, J., Hanna, C. F., Hollar, R., Pepe, C., Genger, K., Brentz, C., Murphy, B., Abunimeh, N., Carver, R., Harder, P., Coleman, C., Robertson, E., and Wolf-Rogers, J. (1991b). Abbott Prism: A multichannel heterogeneous chemiluminescence immunoassay analyzer. *Clin. Chem.* **37**, 1540-1547.
- Kricka, L. J. (1991). Chemiluminescent and bioluminescent techniques. *Clin. Chem.* **37**, 1472-1481.
- Kricka, L. J. (1992). Nucleic acid hybridization test formats: Strategies and applications. In "Nonisotopic DNA Probe Techniques" (L. J. Kricka, ed.), pp. 3-28. Academic Press, San Diego.
- Kricka, L. J., and Carter, T. J. N. (1982). Luminescent immunoassays, In "Clinical and Biochemical Luminescence" (L. J. Kricka and T. J. N. Carter, eds.), pp. 153-178. Marcel Dekker, New York.
- Kricka, L. J., Thorpe, G. H. G., and Stott, R. A. W. (1987). Enhanced chemiluminescence enzyme immunoassay. *Pure Appl. Chem.* **59**, 651-654.
- Kuroda, N., Nakashima, K., Akiyama, S., Shiraki, H., and Maeda, Y. (1992). Photographic chemiluminescent ELISA for detection of anti-human T-cell leukemia virus type-I antibodies by using synthetic peptides as antigens. *Clin. Chim. Acta* **211**, 113-119.
- Martin, C. S., and Bronstein, I. (1993). Imaging of chemiluminescent signals with cooled CCD camera systems. Presented at the 2nd European Seminars on Low Light Imaging, Florence, Italy, September 1-4.
- Martin, C. S., and Bronstein, I. (1994). Imaging of chemiluminescent signals with cooled CCD camera systems. *J. Biolumin. Chemilumin.* **9**, 145-153.
- Martinez, M. L., and Weiss, R. C. (1993). Detection of feline infectious peritonitis virus infection in cell cultures and peripheral blood mononuclear leukocytes of experimentally infected cats using a biotinylated cDNA probe. *Vet. Microbiol.* **34**, 259-271.
- Matthews, J. A., Batki, A., Hynds, C., and Kricka, L. J. (1985). Enhanced chemiluminescent method for the detection of DNA dot-hybridization assays. *Anal. Biochem.* **151**, 205-209.
- McCapra, F. (1966). The chemiluminescence of organic compounds. *Q. Rev. Chem. Soc.* **20**, 485-510.
- McCartney, R. A., Harbour, J., Roome, A. P. C. H., and Caul, E. O. (1993). Comparison of enhanced chemiluminescence and microparticle enzyme immunoassay for the measurement of hepatitis-B surface antibody. *Vaccine* **11**, 941-945.
- McKimm-Breschkin, J. L. (1992). Rapid treatment of whole cells and RNA viruses for analysis of RNA slot blot hybridization. *Virus Res.* **22**, 199-206.
- Mercer, L. J., Robinson, D. C., Sahm, D. F., Lawrie, M. J., and Hajj, S. N. (1990). Comparison of chemiluminescent DNA probe to cell culture for the screening of *Chlamydia trachomatis* in a gynecology clinic population. *Obstet. Gynecol.* **76**, 114-117.
- Miliukiene, V., Dikiniene, N., Vidziunaite, R., Mikalauskiene, G., Veleckaite, A., and Mikulskis, P. (1991). The comparative evaluation of spectrophotometric and chemiluminescent detection in an immunoenzyme test of antibodies to the antigens of the bovine leukosis virus. *Zh. Mikrobiol. Epidemiol. Immunobiol.* **7**, 64-66.
- Musiani, M., Zerbini, M., Gibellini, D., Gentilomi, G., La Placa, M., Ferri, E., and Girotti, S. (1991a). Chemiluminescent assay for the detection of viral and plasmid DNA using dogoxigenin-labeled probes. *Anal. Biochem.* **194**, 394-398.
- Musiani, M., Zerbini, M., Gibellini, D., Gentilomi, G., Venturoli, S., Gallinella, G., Ferri, E., and Girotti, S. (1991b). Chemiluminescence dot blot hybridization assay for detection of B19 parvovirus DNA in human sera. *J. Clin. Microbiol.* **29**, 2047-2050.
- Musiani, M., Zerbini, M., Gentilomi, G., Gibellini, D., Gallinella, G., Venturoli, S., and La Placa, M. (1992). Detection of CMV DNA in clinical samples of AIDS patients by chemiluminescence by hybridization. *J. Virol. Meth.* **38**, 1-9.
- Nelson, N. C., and Kacian, D. L. (1990). Chemiluminescent DNA probes: A comparison of



- the acridinium ester and dioxetane detection system and their use in clinical diagnostic assays. *Clin. Chim. Acta* **194**, 73-90.
- Nelson, N. C., Reynolds, M. A., and Arnold, L. J. Jr. (1992). Detection of acridinium esters by chemiluminescence. In "Nonisotopic DNA Probe Techniques" (L. J. Kricka, ed.), pp. 275-310. Academic Press, San Diego.
- Neman-Simha, V., Delmas-Beauvieux, M. C., Geniaux, M., and Bebear, C. (1991). Evaluation of a chemiluminometric immunoassay and a direct immunofluorescence test for detecting *Chlamydia trachomatis* in urogenital specimens. *Eur. J. Clin. Microbiol. Infect. Dis.* **10**, 662-665.
- Nicolas, J. C., Térouanne, B., Balaguer, P., Boussioux, A. M., and Crastes de Paulet, A. (1990). A bioluminescent solid phase for immunoassays and DNA probes. *Ann. Biol. Clin. (Paris)* **48**, 573-579.
- Nicolas, J.-C., Balaguer, P., Térouanne, B., Villebrun, M. A., and Boussioux, A.-M. (1992). Detection of glucose 6-phosphate dehydrogenase by bioluminescence. In "Nonisotopic DNA Probe Techniques" (L. J. Kricka, ed.), pp. 203-225. Academic Press, San Diego.
- O'Neill, H. J., and Coyle, P. V. (1992). Two anti-parvovirus B19 IgM capture assays incorporating a mouse monoclonal antibody specific for B19 viral capsid proteins Vp1 and Vp2. *Arch. Virol.* **123**, 125-134.
- Ou, C.-Y., McDonough, S. H., Cabanas, D., Ryder, T. B., Harper, M., Moore, J., and Schochetman, G. (1990). Rapid and quantitative detection of enzymatically amplified HIV-1 DNA using chemiluminescent oligonucleotide probes. *AIDS Res. Hum. Retroviruses* **6**, 1323-1329.
- Papsidero, L. D., Dittmer, R. P., Vaickus, L., and Poiesz, B. J. (1992). Monoclonal antibodies and chemiluminescence immunoassay for detection of the surface protein of human T-cell lymphotropic virus. *J. Clin. Microbiol.* **30**, 351-358.
- Podleckis, E. V., Hammond, R. W., Hurtt, S. S., and Hadidi, A. (1993). Chemiluminescent detection of potato and pome fruit viroids by dogoxigenin-labeled dot blot and tissue blot hybridization. *J. Virol. Meth.* **43**, 147-158.
- Pollard-Knight, D., Read, C. A., Downes, M. J., Howard, L. A., Leadbetter, M. R., Pheby, S. A., McNaughton, E., Syms, A., and Brady, M. A. W. (1990a). Nonradioactive nucleic acid detection by enhanced chemiluminescence using probes directly labeled with horseradish peroxidase. *Anal. Biochem.* **185**, 84-89.
- Pollard-Knight, D., Simmonds, A. C., Schaap, A. P., Akhavan, H., and Brady, M. A. W. (1990b). Nonradioactive DNA detection on Southern blots by enzymatically triggered chemiluminescence. *Anal. Biochem.* **185**, 353-358.
- Pollini, C. P., Giunchedi, L., and Credi, R. (1993). A chemiluminescent immunoassay for the diagnosis of grapevine closteroviruses on nitrocellulose membrane. *J. Virol. Meth.* **42**, 107-116.
- Pronovost, A. D., Baumgarten, A., and Hsiung, G. D. (1981). Sensitive chemiluminescent enzyme-linked immunosorbent assay for quantification of human immunoglobulin G and detection of herpes simplex virus. *J. Clin. Microbiol.* **13**, 97-101.
- Puchhammerstoedckl, E., Heinz, F. X., and Kunz, C. (1993). Evaluation of 3 nonradioactive DNA detection systems for identification of herpes simplex DNA amplified from cerebrospinal fluid. *J. Virol. Meth.* **43**, 257-266.
- Radziszewski, B. (1877). Untersuchungen über Hydrobenzamid, Amarin und Lophin. *Chem. Berlin* **10**, 70-75.
- Rapier, J. M., Villamarzo, Y., Schochetman, G., Ou, C. Y., Brakel, C. L., Donegan, J., Maltzman, W., Lee, S., Kirtikar, D., and Gatica, D. (1993). Nonradioactive colorimetric microplate hybridization assay for detecting amplified human immunodeficiency virus DNA. *Clin. Chem.* **39**, 244-247.
- Robertson, E. F., Weare, J. A., Randell, R., Holland, P. V., Madsen, G., and Decker, R. H. (1991). Characterization of a reduction-sensitive factor from human plasma responsible

- for apparent false activity in competitive assays for antibody to hepatitis B core antigen. *J. Clin. Microbiol.* **29**, 605–610.
- Sarkar, F. H., Sakr, W. A., Li, Y. W., Sreepathi, P., and Crissman, J. D. (1993). Detection of human papillomavirus (HPV) DNA in human prostatic tissues by polymerase chain reaction (PCR). *Prostate* **22**, 171–180.
- Schmidt, B. L. (1991). A rapid chemiluminescence detection method for PCR-amplified HIV-1 DNA. *J. Virol. Meth.* **32**, 233–244.
- Schmidt, B. L., and Gschnait, F. (1991). Detection of causal agents of AIDS using polymerase chain reaction and chemiluminescence measurement. *Hautarzt* **42**, 754–758.
- Scieux, C., Bianchi, A., Henry, S., Brunat, N., Abdennader, S., Vexiau, D., Janier, M., Morel, P., and Lagrange, P. H. (1992a). Evaluation of a chemiluminometric immunoassay for detection of *Chlamydia trachomatis* in the urine of male and female patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **11**, 704–708.
- Scieux, C., Bianchi, A., Vassias, I., Meouchy, R., Felten, A., Morel, P., and Perol, Y. (1992b). Evaluation of a new chemiluminometric immunoassay, Magic Lite *Chlamydia*, for detecting *Chlamydia trachomatis* antigen from urogenital specimens. *Sex. Transm. Dis.* **19**, 161–164.
- Septak, M. (1989). Acridinium ester-labelled DNA oligonucleotide probes. *J. Biolumin. Chemilumin.* **4**, 351–356.
- Simmonds, A. C., Cunningham, M., Durrant, I., Fowler, S. J., and Evans, M. R. (1991). Enhanced chemiluminescence in filter-based DNA detection. *Clin. Chem.* **37**, 1527–1528.
- Sritharan, V., and Barker, R. H., Jr. (1991). A simple method for diagnosing *M. tuberculosis* infection in clinical samples using PCR. *Mol. Cell. Probes* **5**, 385–395.
- Stanley, P. E. (1992a). A survey of more than 90 commercially available luminometers and imaging devices for low light measurements of chemiluminescence and bioluminescence, including instruments for manual, automatic and specialized operation, for HPLC, LC, GLC and microtitre plates. Part 1: Descriptions. *J. Biolumin. Chemilumin.* **7**, 77–108.
- Stanley, P. E. (1992b). A survey of more than 90 commercially available luminometers and imaging devices for low light measurements of chemiluminescence and bioluminescence, including instruments for manual, automatic and specialized operation, for HPLC, LC, GLC and microtitre plates. Part 2: Photographs. *J. Biolumin. Chemilumin.* **7**, 157–169.
- Stanley, P. E. (1993a). A survey of some commercially available kits and reagents which include bioluminescence or chemiluminescence for their operation—including immunoassays, hybridization, labels, probes, blots and ATP-based rapid microbiology: Products from more than 40 companies. *J. Biolumin. Chemilumin.* **8**, 51–63.
- Stanley, P. E. (1993b). Commercially available luminometers and imaging devices for low-light measurements and kits and reagents utilizing bioluminescence or chemiluminescence: Survey update I. *J. Biolumin. Chemilumin.* **8**, 237–240.
- Stauber, S., Siegel, G., Janitschke, K., Schulze-Forster, K., and Simon, D. (1991). Detection of polymerase chain reaction (PCR) products of *Toxoplasma gondii* by enhanced chemiluminescence (ECL). *J. Biolumin. Chemilumin.* **6**, 283.
- Suzuki, K., Okamoto, N., Watanabe, S., and Kano, T. (1992). Chemiluminescent microtiter method for detecting PCR amplified HIV-1 DNA. *J. Virol. Meth.* **38**, 113–122.
- Suzuki, K., Craddock, B. P., Kano, T., and Steigbigel, R. T. (1993). Chemiluminescent enzyme-linked immunoassay for reverse transcriptase, illustrated by detection of HIV reverse transcriptase. *Anal. Biochem.* **210**, 277–281.
- Tham, K. M., and Stanislawek, W. L. (1992a). Detection of chicken anaemia agent DNA sequences by the polymerase chain reaction. *Arch. Virol.* **127**, 245–255.
- Tham, K. M., and Stanislawek, W. L. (1992b). Polymerase chain reaction amplification for direct detection of chicken anemia virus DNA in tissues and sera. *Avian Dis.* **36**, 1000–1006.
- Thorpe, G. H. G., Kricka, L. J., Moseley, S. B., and Whitehead, T. P. (1985). Phenols as enhancers of the chemiluminescent horseradish peroxidase-luminol-hydrogen peroxide

- reaction: Application in luminescence-monitored enzyme immunoassays. *Clin. Chem.* **31**, 1335-1341.
- Tumolo, A., Nguyen, Q., Witney, F., Murphy, O. J., Voyta, J. C., and Bronstein, I. (1992). Detection of DNA on membranes with alkaline phosphatase-labeled probes and chemiluminescent AMPPD substrate. In "Nonisotopic DNA Probe Techniques" (L. J. Kricka, ed.), pp. 127-145. Academic Press, San Diego.
- Urdea, M. S., Running, J. A., Horn, T., Clyne, J., Ku, L., and Warner, B. D. (1987) A novel method for the rapid detection of specific nucleotide sequences in crude biological samples without blotting or radioactivity; application to the analysis of hepatitis B virus in human serum. *Gene* **61**, 253-264.
- Urdea, M. S., Kolberg, J., Clyne, J., Running, J. A., Besemer, D., Warner, B., and Sanchez-Pescador, R. (1989). Application of a rapid non-radioisotopic nucleic acid analysis system to the detection of sexually transmitted disease-causing organisms and their associated antimicrobial resistances. *Clin. Chem.* **35**, 1571-1575.
- Urdea, M. S., Kolberg, J., Warner, B. D., Horn, T., Clyne, J., Ku, L., and Running, J. A. (1990). A novel method for the rapid detection of hepatitis B virus in human serum samples without blotting or radioactivity. In "Luminescence Immunoassay and Molecular Applications" (K. van Dyke and R. van Dyke, eds.), pp. 275-292. CRC Press, Boca Raton, Florida.
- Vlaspolder, F., Matsaers, J. A., Blog, F., and Notowicz, A. (1993). Value of a DNA probe assay (Gen-Probe) compared with that of culture for diagnosis of gonococcal infection. *J. Clin. Microbiol.* **31**, 107-110.
- Vlieger, A. M., Medenblik, A. M., van-Gijlswijk, R. P., Tanke, H. J., van-der-Ploeg, M., Gratama, J. W., and Raap, A. K. (1992). Quantitation of polymerase chain reaction products by hybridization-based assays with fluorescent, colorimetric, or chemiluminescent detection. *Anal. Biochem.* **205**, 1-7.
- Wages, J. M., Dolenga, L., and Fowler, A. K. (1993). Electrochemiluminescence detection and quantitation of PCR-amplified DNA. *Amplifications* **10**, 1-3.
- Weare, J. A., Robertson, E. F., Madsen, G., Hu, R., and Decker, R. H. (1991). Improvement in the specificity of assays for detection of antibody to hepatitis B core antigen. *J. Clin. Microbiol.* **29**, 600-604.
- Weeks, I., Behesti, I., McCapra, F., Campbell, A. K., and Woodhead, J. S. (1983). Acridinium esters as high-specific-activity labels in immunoassay. *Clin. Chem.* **29**, 1474-1479.
- Welnicki, M., and Hiruki, C. (1993). Chemiluminescent assay for the detection of viral and viroid RNAs using digoxigenin labelled probes. *J. Biolumin. Chemilumin.* **8**, 127.
- Whitehead, T. P., Thorpe, G. H. G., Carter, T. J. N., Groucutt, C., and Kricka, L. J. (1983). Enhanced luminescence procedure for sensitive determination of peroxidase-labelled conjugates in immunoassay. *Nature* **305**, 158-159.
- Yang, J. Q., Tata, P. V., Park-Turkel, H. S., and Waksal, H. W. (1991). The application of AmpliProbe in diagnostics. *BioTechniques*. **11**, 392-397.
- Zachar, V., Mayer, V., Aboagye-Mathiesen, G., Norskov-Laruitzen, N., and Ebbesen, P. (1991). Enhanced chemiluminescence-based hybridization analysis for PCR-mediated HIV-1 DNA detection offers an alternative to <sup>32</sup>P-labelled probes. *J. Virol. Meth.* **33**, 391-395.

# Neurotoxicology *In Vitro*

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- XIA, Z., DICKENS, M., RAINGAUD, J., DAVIS, R.J. & GREENBURG, M.E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, 270, 1326-1331.
- ZIELKE, H.R., JACKSON, M.J., TILDON, J.T. & MAX, S.R. (1993). A glutamatergic mechanism for aluminium toxicity in astrocytes. *Molecular and Chemical Neuropathology*, 19, 219-233.
- ZHANG, J., DAWSON, V.L., DAWSON, T.M. & SNYDER, S.H. (1994). Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. *Science*, 263, 687-389.

# 8

## Cell Contact and Cell Communication

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### Contents

- 8.1 Introduction
- 8.2 Use of cell culture and tissue slice preparations for the assessment of gap junctional intercellular communication
- 8.3 Dye coupling vs electrophysiological coupling
- 8.4 Methodological considerations
- 8.5 Conclusions
- 8.6 References

## 8.1 INTRODUCTION

Interaction and cooperation between cells in an organ allow for an organism to function as a coordinated living entity. There are several means by which cells can communicate with each other (Figure 8.1). Cells not directly in contact with each other or at distant sites from each other in the body can communicate through hormonal interaction via the blood. This hormonal regulation is mediated by the specific receptors unique to the target cell. Locally, cells also communicate through cell surface glycoproteins or adhesion molecules such as cadherins, integrins, or members of the immunoglobulin superfamily and thereby exchange information between cells and the extracellular matrix (Bell, 1978; Jessell, 1988; Hynes, 1992). Another important form of communication between two adjacent cells is through the gap junction, which is a surface organelle (Revel *et al.*, 1967) composed of two hemichannels (connexons), one from each cell. Gap junctions function as a conduit for the exchange of small water-soluble molecules and electrotonic transmission between excitable cells. The substances that have been demonstrated to cross gap junctions include amino acids, sugars, nucleotides, and second messengers such as cAMP and calcium (Rieske *et al.*, 1975; Pitts and Sims, 1977; Tsien and Weingart, 1976; Cornell-Bell *et al.*, 1990). By allowing these information changes, gap junctions facilitate the synchronization of electronic and metabolic cooperation, which appears to be required for the correct functioning of the cells, including maintenance of homeostasis, normal embryogenesis, control of cell proliferation, and maintenance of cell differentiation (Caveney, 1985; Loewenstein, 1981; Rose and Rick, 1978; Chang *et al.*, 1987).

Due to its important role in normal cell physiology, disruption of gap junctional intercellular communication has been postulated to be associated with a variety of pathological and toxicological conditions and disease states including carcinogenesis (Klaunig and Ruch, 1990), teratogenesis (Warner *et al.*, 1984), neurotoxicity (Trosko *et al.*, 1987), reproductive dysfunction (Ye *et al.*, 1990), cardiovascular diseases (Kleber *et al.*, 1987), ischaemia (Smith *et al.*, 1991) and cholestasis (Traub *et al.*, 1983).

In recent years, additional interest has been seen in defining the role of cell-to-cell communication through gap junctions in the nervous system. The diversity of cell types in the nervous system impacts on the type and degree of intercellular communication seen. Through their intercellular interactions these diverse cell types form functional interacting compartments within the brain. The degree of intercellular communication within each cell population varies according to cell type, and there is evidence that communication also occurs between different cell types. It has been generally held that chemical transmission is the major mechanism by which neuronal cells interact. However, more recently, morphological and electrophysiological evidence suggests the existence of extensive interneuronal gap junctional interaction. Electrical coupling through gap junctions in the nervous system may constitute an important mode of neuronal intercellular communication and contribute significantly to the integrative properties of the neuronal

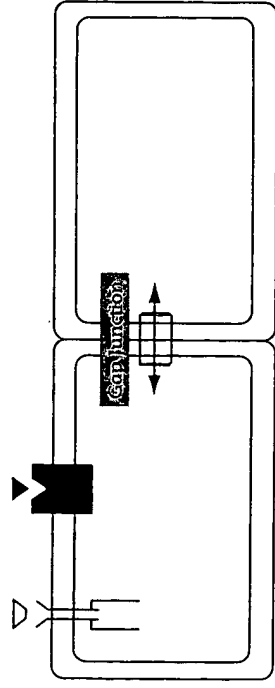
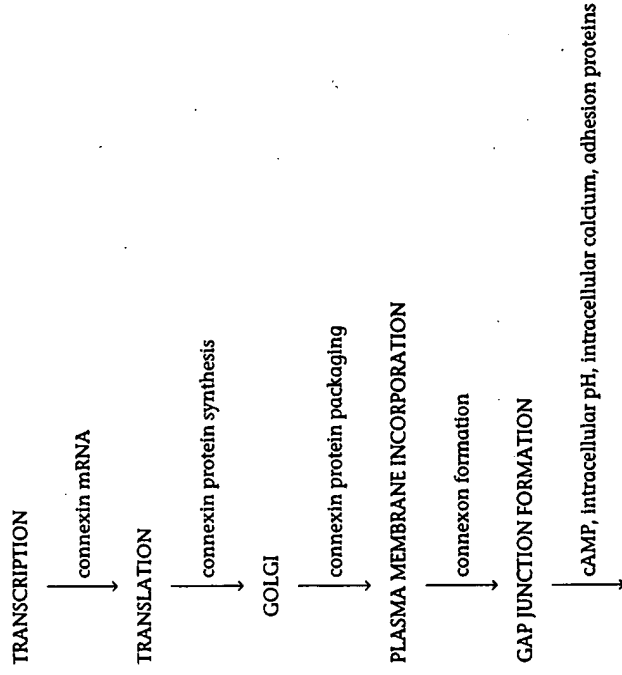


Figure 8.1: Modalities of intercellular communication. Hormones (▼) and growth factors (▽) are transported to the targets via blood. Ions and small molecules (< 1000 Da) can pass through gap junctions from one cell to its neighbours and influence the function of the connecting cells.

cells. It is speculated that cell-to-cell coupling in the immature neocortex defines an important microenvironment to guide the formation of neuronal synapse (Peinado *et al.*, 1993). When it is maturing, gap junctional intercellular communication, as indicated by dye coupling, declines in neocortex, thus correlating inversely with the differentiation of the brain (Peinado *et al.*, 1993; Connors *et al.*, 1983). The possible roles of gap junctional intercellular communication between mature neurons have been discussed previously by Dermietzel and Spray (1993). Compared with neurons, gap junctions are more common in glial cells, especially in astrocytes. The extensive distribution of gap junctions and the resulting gap junctional intercellular communication seen between astrocytes give rise to an astrocytic syncytium which appears to function in the rapid redistribution of potassium ions after neuronal firing (Gardner-Medwin, 1983). The transfer and distribution of calcium and inositol triphosphate have also been suggested as a function of astrocytic gap junctional intercellular communication (Cornell-Bell *et al.*, 1990; Kim *et al.*, 1994). In C6 glioma cells gap junctions may propagate and amplify ischemic injury (Lin *et al.*, 1998). It can be concluded that gap junction mediated intercellular communication is an important means of buffering electrolytes and maintaining homeostasis in the astrocyte cell population in the CNS.

Its ubiquitous nature and important physiological role make the gap junction a target for the toxic effects of exogenous chemicals. It is of great help to understand the mechanisms of gap junction regulation before discussion of toxic effects. Several molecular intracellular steps must take place before gap junctions can be formed and function (Figure 8.2). First, mRNAs for different connexins are transcribed in the nucleus, and transported to cytoplasm for translation. Connexin protein is produced in rough endoplasmic reticulum and assembled in the Golgi. Six connexin proteins constituting a hemi-connexon structure are in turn inserted into the plasma membrane. Two hemi-connexons from adjacent cells are connected to form a channel. A collection of these conduits results in the formation of the gap junction membrane structure (Revel and Karnovsky, 1967). The correct orientation of the two hemichannels appears to be influenced by adhesion molecule mediated



#### FUNCTIONAL GAP JUNCTIONAL COMMUNICATION

Figure 8.2: Molecular and intracellular control of gap junction formation and function

cell contacts (Kanno *et al.*, 1984; Mege *et al.*, 1988). After being successfully assembled, the function (intercellular communication) of the gap junction is dependent on the regulation and intercellular concentration of a variety of intracellular factors, including protein kinase C (Gainer and Murray, 1985; Hartman and Rosen, 1985), pH (Spray *et al.*, 1986), cAMP (Flagg-Newton *et al.*, 1981; Klaunig and Ruch, 1987) and intracellular calcium (Peracchia and Peracchia, 1980; Rose and Rick, 1978). Modulation of these factors can affect the opening and closing of the gap junction channels and thus regulate intercellular communication. Toxic agents may target any of the stages of gap junction formation from connexin transcription, to protein synthesis, to assembly of the connexon in the plasma membrane, to the modulation of intracellular control of the pore opening and closing.

Since the first discovery of an electron microscopically detected structure, the nexus, extensive literature has appeared describing the structure, function and pathological changes of gap junctions. Along with this is the development of a variety of approaches to investigate these phenomena. Initial studies used electron microscopy and provided important structural information on the gap junction plaque and its components. Robertson (1963) described hexagonally packed subunits in electrical synapses between Mauthner cells in the medulla oblongata of the goldfish brain. Freeze-fracture electron microscopy has further described the relationship of connexin and connexon structures

(Rash and Yasumura, 1992). Revel and Karnovsky (1967) found an intercellular space in heart and liver that measured 1–2 nm in width and allowed tracer molecules to penetrate between cells. In excitable cells such as neurons, myocytes and smooth muscle cells, electrical coupling has been used to assess the function of gap junctions (Bennett and Goodenough, 1978). Metabolic cooperation and fluorescent dye transfer (dye coupling) have been extensively used to assess the functional nature of gap junctions. More recently, antibodies produced against connexin protein have been used to identify gap junction location and concentration in cell by immunocytochemistry (Shiosaka *et al.*, 1989; Yamamoto *et al.*, 1989). Similarly, *in situ* hybridization has been employed to define those cells that produce mRNA for specific gap junctions within a tissue and organ (Matsumoto *et al.*, 1991). While electron microscopy, immunocytochemistry, and *in situ* hybridization can be used to examine the structure and the distribution of gap junction proteins and cells making specific gap junctional proteins, these techniques provide little information of the functionality of the gap junctions in a tissue or organ. Functional assessment of the gap junction, intercellular communication, has been accomplished using electrical coupling, dye transfer, and metabolic cooperation.

## 8.2 USE OF CELL CULTURE AND TISSUE SLICE PREPARATIONS FOR THE ASSESSMENT OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

The examination of gap junctional intercellular communication in a normal situation is difficult, if not impossible. Even though the effects of toxicants on the expression of gap junction mRNA and proteins can be assessed following *in vivo* treatment using molecular and morphologic approaches, little information about the function of gap junctions (intercellular communication) has been retrieved by such studies. Several approaches have been described for the *in vitro* study of gap junctional intercellular communication in the nervous system. These methods basically fall into two types of preparation: tissue slice and cell culture (either primary or secondary). Both dye transfer (coupling) and electro-physiological coupling have been studied in these preparations.

In cell cultures, either primary or secondary, the effect of a compound on gap junctional intercellular communication can be assessed directly by measuring electrophysiological coupling or dye coupling following treatment of the cells with the chemical of interest. Use of primary and secondary cell cultures has provided important information on the mechanisms of action of toxic agents on neural cell gap junctions. In addition, the use of cell cultures provide a relatively easy and repeatable system by which multiple studies can be performed efficiently while also reducing the number of experimental animals involved. However, in culture, cells are more accessible to the toxicant due to the continuous bathing of the cells with the compound and they also lack the three-dimensional

structure seen *in vivo*. Thus, while cell culture approaches provide important information on the mechanisms involved in the interruption of cell-to-cell communication by toxic agents, the exact interaction of toxicants with the brain cells seen *in vivo* cannot be duplicated using a two-dimensional culture approach. In addition, when preparing and using cell cultures for gap junctional communication studies, modification of gap junction expression may occur during the isolation and culture process. This is supported in part by the fact that the incidence of dye coupling in neurons is lower in culture than in the hippocampal slice preparations (20 vs 50 per cent) (O'Beirne *et al.*, 1987).

Compared with cell cultures, tissue slice preparations have several advantages including that the normal three-dimensional intercellular contact and interaction seen in the brain are more closely maintained and the effect of *in vivo* treatment on the brain gap junctions can be more accurately determined. Tissue slice preparations also have disadvantages including a restriction in the number of replicates and various experimental manipulations (dose response) that can be performed in a timely manner. In addition, the evaluation of cell-to-cell communication using the slice techniques involves a greater amount of technical skill and effort.

## 8.3 DYE COUPLING VS ELECTROPHYSIOLOGICAL COUPLING

Dye coupling is the phenomenon of dye movement from one cell to an adjacent contacting neighbouring cell through the gap junction (connexon). The molecular weight cutoff for materials that traverse through the gap junction has been estimated to be less than 1000 Da for cells (Flagg-Newton *et al.*, 1979). Therefore, to assess gap junction function in mammalian cells using dye transfer (coupling), a molecular marker of less than 1 kDa must be used. In addition, probes for junctional permeability should have the following properties: strongly hydrophilic, impermeable through non-junctional membrane, low toxicity, and high sensitivity upon detection. The first fluorescent probe used to explore gap junction was fluorescein (Loewenstein and Kanno, 1964). However, this probe also displayed permeability through non-junctional membranes, which has prevented its further utilization. Other fluorescent dyes such as Procion yellow M4RS have been used in neuronal cell staining (Stretton and Kravitz, 1968). However, the low fluorescent efficiency of Procion yellow as well as its toxic effects in some cells has restricted its use (Payton *et al.*, 1969). In 1978, Stewart described and characterized a new fluorescent dye, Lucifer yellow CH. This dye has the properties noted above for use in gap junctional function evaluation. It is sensitive (intense fluorescence), has low toxicity, is not permeable through non-junctional membranes, rapidly spreads to the adjacent cells, and is retained during tissue fixation. These major advantages make Lucifer yellow CH a popular tool for testing gap junctional intercellular communication in a variety of cells, including neurons and astrocytes.

In our laboratory, Lucifer yellow CH has been used as a tracer for quantitative and qualitative evaluation of gap junctional intercellular communication in primary and



**TABLE 8.1:**  
Effects of acrylonitrile (ACN) on gap junctional intercellular communication in rat astrocytes

Dose of ACN (M)	Duration of exposure (hours)		
	2	4	24
0	97.67 ± 1.4	94.64 ± 4.1	94.64 ± 4.0
1 × 10 <sup>-5</sup>	94.34 ± 2.0	89.86 ± 2.5 <sup>a</sup>	84.63 ± 9.1 <sup>b</sup>
1 × 10 <sup>-4</sup>	89.34 ± 1.6 <sup>a</sup>	76.29 ± 9.7 <sup>b</sup>	77.33 ± 18.2 <sup>b</sup>
1 × 10 <sup>-3</sup>	83.18 ± 1.1 <sup>b</sup>	60.72 ± 6.2 <sup>b</sup>	63.43 ± 16.3 <sup>b</sup>

Values represent the mean ± the SD percentage of dye coupled astrocytes with  $n > 3$ .

<sup>a</sup> Statistical significance from untreated control to  $P < 0.01$  by Fisher exact test.

<sup>b</sup> Statistical significance from untreated control to  $P < 0.0001$  by Fisher exact test.

secondary cell cultures. Using Lucifer yellow, the effect of toxic agents on cell-to-cell communication can be examined in defined doses and time-dependent relationships following toxicant treatment. We have examined, for example, the effect of the neurocarcinogen, acrylonitrile, on gap junctional intercellular communication in a rat astrocyte cell line (DI TNC1 cell line). Acrylonitrile is a chemical monomer widely used in industry that induces astrocytomas in a dose response manner in rat brain following chronic exposure (Bigner *et al.*, 1986). Acrylonitrile appears to function through non-genotoxic carcinogenic mechanisms, possibly through the induction of oxidative damage (oxidative stress). Blockage of cell-to-cell communication appears to be a characteristic of most non-genotoxic carcinogens and has also been seen following exposure to reactive oxygen species. Acrylonitrile inhibited gap junctional intercellular communication in rat astrocytes in a dose-dependent manner (Table 8.1). This inhibition was prevented by the cotreatment with vitamin E, a well known antioxidant (Table 8.2). These data confirm a role of oxidative stress in acrylonitrile-induced inhibition of gap junctional intercellular communication in rat astrocytes, and correlate with previous *in vitro* and *in vivo* observations by our group which showed acrylonitrile-induced oxidative stress in both rat brain and rat astrocytes (J. Jiang and J.F. Klaunig, unpublished data).

Electrical coupling is the capacity for passive (electronic) spread of a transient electrical potential from a cell to an adjacent contiguous cell (Socolar and Loewenstein, 1979). When pulses of hyperpolarizing or hypopolarizing current are passed from cell to cell with the aid of intracellular microelectrodes, the detection of the voltage deflection in both the stimulated cell and the communicating neighbour cell can be made. This transmission of electrical coupling potential is bidirectional, and the gap junction has been demonstrated as the structural basis for this electrical transmission (Peracchia, 1980; Loewenstein, 1981). Although this method is sensitive in indicating gap junctional permeability, the procedure is extremely time-consuming and difficult (Rao *et al.*, 1986). The other problem

**TABLE 8.2:**

Effects of  $\alpha$ -tocopherol (Vit E) on acrylonitrile (ACN) induced inhibition of gap junctional communication in rat astrocytes

Dose of ACN (M)	Duration of exposure (hours)		
	4	4 + 10 <sup>-4</sup> M Vit E	24
0	91.69 ± 5.2	94.38 ± 3.9	94.59 ± 5.0
1 × 10 <sup>-5</sup>	89.99 ± 3.4	89.84 ± 3.3	86.33 ± 5.0 <sup>a</sup>
1 × 10 <sup>-4</sup>	84.88 ± 0.9 <sup>a</sup>	86.03 ± 8.5	83.80 ± 2.3 <sup>c</sup>
1 × 10 <sup>-3</sup>	62.37 ± 5.8 <sup>a,c</sup>	84.22 ± 10.1 <sup>a,b,d</sup>	68.80 ± 4.5 <sup>c</sup>

Values represent the mean ± the SD (% recovery) percentage of dye coupled astrocytes with  $n > 3$ .

<sup>a</sup> Statistical significance from untreated control to  $P < 0.05$  by Fisher exact test.

<sup>b</sup> Statistical significance from respective treatment group to  $P < 0.05$  by Fisher exact test.

<sup>c</sup> Statistical significance from untreated control to  $P < 0.01$  by Fisher exact test.

<sup>d</sup> Statistical significance from respective treatment group to  $P < 0.01$  by Fisher exact test.

encountered using electrophysiological methodology is the difficulty in selecting a coupled pair of neurons. Because the coupling is not always somatic to somatic, two neurons might be apart from each other but still connected through gap junctions in the dendrites. These technical problems make dye-coupling a more attractive approach for the effects of toxicants on nervous system gap junctional intercellular communication.

## 8.4 METHODOLOGICAL CONSIDERATIONS

A number of culture systems, discussed extensively in Chapter 10, are available for the analysis of intercellular communication via gap junctions. In the following sections we briefly summarize some specific procedures successfully employed in this laboratory.

### 8.4.1 Cell culture: rat hippocampal neurons and rat astrocyte cell line (DI TNC1)

Hippocampi from 18-day-old gestational rats are dissected, dissociated and cells plated onto polylysine-coated coverslips by the standard methods (see O'Beirne *et al.*, 1987). Coverslips with neurons are transferred to a recording chamber on an inverted microscope and are perfused with oxygenated (5% CO<sub>2</sub>-95% O<sub>2</sub>) artificial CSF (Knut and Westgaard, 1971). Neurons are easily recognized because their cell bodies are bright by phase contrast microscopy and are raised above the glial monolayer.

Rat astrocytes (DI TNC1) are plated at 1 × 10<sup>6</sup> cells per 60 mm culture dish. Cells are cultured in DMEM/F12 medium containing 4.5 g/l glucose, 10% FBS and 10 ml of Pen/Strep solution. Cultures are incubated at 37°C and 5% CO<sub>2</sub> for 24 hours prior to treatment.

## 8.4.2 Loading and detection of fluorescent dye coupling

Loading dye into cells can be accomplished by several means, including microinjection (Stewart, 1978) and scrape-loading (El-Fouly *et al.*, 1987). Because it is easy to use and easy to control, microinjection with iontophoresis is the most popular technique in studying gap junctions in nervous system in both slice preparation and cell culture. We also focus on this method in this review (Klaunig and Baker, 1994).

Microelectrode injection needles are pulled from 1.5 mm diameter single-barrel glass capillaries. The tip opening diameter is measured through tip resistance to be 1  $\mu\text{m}$ . Using a binocular dissecting scope, the pipettes are initially loaded with 5  $\mu\text{l}$  of 5% Lucifer yellow CH and backfilled with LiCl. The injection pipettes must be made fresh before each injection period. The injection micropipette is located in the microscope field of view at 200 $\times$  and manoeuvred slowly to the cell of interest using the micromanipulators until the tip pierces the plasma membrane at a glancing angle. A current of 3 nA is then applied into the micropipette, forcing the Lucifer yellow CH into the cell cytoplasm.

In primary culture, impalements are monitored throughout the injections to ensure that neurons remain excitable and membrane potential is stable. Live cells injected with Lucifer yellow are visualized in the recording chamber using epifluorescence and photographed immediately. The  $\chi^2$  test for homogeneity is used to compare the incidence of dye coupling between groups (O'Beirne *et al.*, 1987).

In secondary cell culture, the cell that is injected with Lucifer yellow (referred to as the donor cell) is viewed with the fluorescence microscope and the amount of dye coupling is scored. Dye coupling is quantitated by counting both the number of cells (recipients) that contact the donor cell that express dye coupling to them and the number of recipients that do not show dye coupling with the donor cell. The percentage of dye coupling is determined by dividing the number of coupled recipients by the total number of recipients (both coupled and uncoupled). Enough donor cells are microinjected so that 50–75 recipients can be evaluated per culture dish. Triplicate dishes should be examined for each concentration and duration of toxicant examined (Klaunig and Baker, 1994).

## 8.4.3 Electrophysiological coupling

Intracellular recordings are obtained in culture using conventional microelectrodes. Impalements are monitored throughout the injections to ensure that neurons remain excitable and membrane potential is stable. Simultaneous intracellular recordings are obtained from adjacent neurons to test for electronic coupling. The  $\chi^2$  test for homogeneity is used to compare the incidence of dye coupling between groups (O'Beirne *et al.*, 1987).

## 8.4.4 Tissue slices

### 8.4.4.i Preparation of tissue slices

Tissue slices have been successfully prepared from piriform cortex (Richards and Sercombe, 1968), the hippocampus (Schwartzkroin, 1975), neocortex (Gutnick and Prince, 1981). All of the existing procedures follow the same basic principles. The following procedure is based on the version of Yamamoto (1972). The region of interest is isolated from the rest of the brain and placed on a piece of filter paper covered with the standard medium. Sections of brain (about 0.3 mm thick) are prepared by cutting the tissue with a razor blade under binocular microscopic observation. Less than 5 minutes after the animal is killed the slices are placed in a constant-flow incubation chamber on a silk mesh in artificial cerebrospinal fluid (composed of NaCl, 134 mM, KCl, 5 mM,  $\text{KH}_2\text{PO}_4$ , 1.24 mM,  $\text{MgSO}_4$ , 1.3 mM,  $\text{CaCl}_2$ , 0.75 mM,  $\text{NaHCO}_3$ , 16 mM, and glucose, 10mM) saturated with 95% of  $\text{O}_2$  and 5%  $\text{CO}_2$ . The same gas mixture is moistened and passed over the upper surface of the preparation.

### 8.4.4.ii Detection of dye coupling

#### Dye injection

The following method of detecting dye coupling in slices of brain is a modified version of MacVicar and Dudek's (1982). Microelectrode injection needles are pulled as described in section 8.4.2. Using a binocular dissecting scope, the pipettes are initially loaded with 5  $\mu\text{l}$  of 5% Lucifer yellow CH and backfilled with LiCl. Cells are injected with dye by passing constant-current hyperpolarizing pulses of 1 s duration at a rate of one every 2 s. During dye injection, action potentials of the cell are monitored. All recordings are obtained from cells with action potentials >50 mV. Only one cell is injected per slice. To minimize the extracellular concentrations of the stain, injections are immediately terminated when the penetration is lost or has significantly deteriorated. To control for artificial staining from Lucifer yellow leakage, neurons that have been briefly impaled but not iontophoresed are impaled for a varying duration. Only impalements for longer than 15–30 s result in detectable staining, and this staining is faint. Poor impalements obtained while searching for a stable recording are terminated in <10 s and Lucifer yellow leakage into these cells does not cause inadvertent staining.

#### Tissue fixation and recording

The fixative of choice is 4% formaldehyde, in 0.1 M sodium phosphate buffer (pH 7.4). This fixative gives good preservation of tissue and cell structure, and causes only a slight increase in background fluorescence. Five to 10 minutes after injection, the slices are placed in 5% formaldehyde in 0.1 M phosphate buffer for 12–24 hours. The slices are then transferred to buffer containing 30% sucrose. Fixed slices are sectioned at 40  $\mu\text{m}$  on a freezing microtome. Sections are placed on slides, cleared (xylene), and mounted in PDX

(a mixture of polystyrene and butyl phthalate in xylene) or methyl salicylate. The sections are then observed with a fluorescence microscope and photographed (MacVicar and Dudek, 1982).

#### 8.4.4.iii Electrophysiological coupling

This method of assessing electronic transmission is based on that described by Knowles *et al.* (1982). The possible existence of electronic coupling between neurons is examined by simultaneous penetration of two neurons. In order to interpret the data, only healthy penetrations are considered for the final analysis. Criteria for healthy penetrations are: membrane potential greater than 50 mV; input resistance greater than 20 M $\Omega$  (measured with 0.5 nA  $\times$  100 ms hyperpolarization intracellular current pulse); spike width less than 2 ms; a steady train of action potentials in response to a 0.5 nA  $\times$  100 ms depolarizing intracellular current pulse. Intracellular penetrations of neurons are made using visual guidance to place the electrodes in slices for somatic penetration. After establishing a healthy penetration of one neuron, a second microelectrode is aimed as closely as possible at the tip of the first electrode to penetrate a second neuron within 50  $\mu$ m of the first. When two penetrations have been established, current pulses are injected into one neuron while the membrane potential of the other neuron is examined at high gain. Depolarizing pulses (0.5 nA  $\times$  100 ms) are used to elicit a spike train in one neuron, while the membrane potential of the other neuron is carefully examined for evidence of postsynaptic potentials. Similarly, when hyperpolarizing pulses (0.5–1.0 nA  $\times$  100 ms) are injected into the first cell, the membrane potential of the second cell is examined for signs of electronic coupling potentials. The procedure is then switched to test for connections in the opposite direction. The intracellular potentials are amplified using high input impedance, capacity compensated amplifiers with internal bridge circuits for injecting current into the cells while recording. The potentials are displayed on an oscilloscope and stored on FM tape for later analysis and photography. The sensitivity for detection of electronic coupling potentials is estimated to be 1 mV, which would allow detection of cells with coupling coefficients greater than about 0.05 (Knowles *et al.*, 1982).

## 8.5 CONCLUSIONS

Since the identification of the gap junction and its importance in normal cellular physiology, extensive efforts have been made to understand the structure, function, and the regulation of this important cytoplasmic organelle. These efforts have been accompanied by the development of new approaches and methodologies to assess gap junctional intercellular communication. In recent years the role that the gap junction plays in toxicologically induced disease has also received increased attention. The approaches outlined above (dye coupling in cell culture and tissue slices) allow for further evaluation of both normal and toxicant-modified gap junction function.

## 8.6 REFERENCES

- BELL, G.I. (1978). Models for the specific adhesion of cells to cells. *Science*, 200, 618–627.
- BENNETT, M.V.L. (1977). Electrical transmission: a functional analysis and comparison with chemical transmission. In: Kandel (Ed.) *Handbook of Physiology, Section I: The Nervous System, Vol. 1, Cellular Biology of Neurons, Part I*, pp. 375–416, Bethesda, American Physiological Society.
- BENNETT, M.V.L. & GOODENOUGH, D.A. (1975). Gap junctions, electronic coupling and intercellular communication. *Neuroscience Research Programme Bulletin*, 16, 371–486.
- BENNETT, M.V.L. & GOODENOUGH, D.A. (1978). Gap junctions, electronic coupling and intracellular communication. *Neuroscience Research Programme Bulletin*, 16, 373–386.
- BIGNER, D.D., BIGNER, S.H., BURGER, P.C., SHELburne, J.D. and FRIEDMAN, H.S. (1986). Primary brain tumors in Fisher 344 rats chronically exposed to acrylonitrile in their drinking water. *Food and Chemical Toxicology*, 24, 129–137.
- CAVENEY, S. (1985). The role of gap junctions in development. *Annual Review of Physiology*, 47, 319–35.
- CHANG, C.C., TROSKO, J.E., EL-FOULY, M.H., GIBSON-D'AMBROSIO, R.E. & D'AMBROSIO, S.M. (1987). *Cancer Research*, 47, 1634–1645.
- CONNORS, B.W., BENARDO, L.S. & PINCE, D.A. (1983). Coupling between neurons of the developing rat neocortex. *Journal of Neuroscience*, 3, 773–782.
- CORNELL-BELL, A.H., FINKBEINER, S.M., COOPER, M.S. & SMITH, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science*, 247, 470–473.
- DERMIETZEL, R. & SPRAY, D.C. (1993). Gap junctions in the brain: where, what type, how many, and why? *Trends in Neuroscience*, 16, 186–192.
- EL-FOULY, M.H., TROSKO, J.E. & CHANG, C.C. (1987). Scrape-loading and dye transfer: a rapid and simple technique to study gap junctional intercellular communication. *Experimental Cell Research*, 168, 422–430.
- FLAGG-NEWTON, J.L., DAHL, G. & LOWENSTEIN, W.R. (1981). Cell junction and cyclic AMP: I. Up regulation of junctional membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. *Journal of Membrane Biology*, 63, 105–121.
- FLAGG-NEWTON, J.L., SIMPSON, I. & LOWENSTEIN, W.R. (1979). Permeability of the cell-to-cell membrane channels in mammalian cell junction. *Science*, 205, 404–407.
- GARNER, H.S.C. & MURRAY, A.W. (1985). Diacylglycerol inhibits gap junctional communication in cultured epidermal cells: evidence for a role of protein kinase C. *Biochemical Biophysical Research Communications*, 126, 1109–1113.
- GARDNER-MEDWIN, A.R. (1983). Analysis of potassium dynamics in mammalian brain tissue. *Journal of Physiology*, 335, 393–426.

- GUTNICK, M.J. & PRINCE, D.A. (1981). Dye coupling and possible electronic coupling in the guinea pig neocortical slice. *Science*, **211**, 67-70.
- HARTMAN, T.G. & ROSEN, J.D. (1985). The effects of some experimental parameters on the inhibition of metabolic cooperation by phorbol myristate acetate. *Carcinogenesis*, **6**, 1315-1319.
- HYNES, R.O. (1992). Integrins: versatility, modulation, and signaling cell adhesion. *Cell*, **69**, 11-25.
- JESSELL, T.M. (1988). Adhesion molecules and the hierarchy of neural development. *Neuron*, **1**, 3-13.
- KANNO, Y., SASAKI, Y., SHIBA, Y., YOSHIDA-NORO, C. & TAKEICHI, M. (1984). Monoclonal antibody ECCD-1 inhibits intercellular communication in teratocarcinoma PCC3 cells. *Experimental Cell Research*, **152**, 270-274.
- KIM, W.T., RIOULT, M.G. & CORNELL-BELL, A.H. (1994). Glutamate-mediated calcium signaling in astrocytes. *Glia*, **11**, 173-184.
- KLAUNIG, J.E. & BAKER, T.B. (1994). Morphological evaluation of gap junctional intercellular communication. In: *Methods in Toxicology*, Vol. 1B, pp. 72-81, San Diego, Academic Press.
- KLAUNIG, J.E. & RUCH, R.J. (1987). Role of cyclic AMP in the inhibition of mouse hepatocyte intercellular communication by liver tumor promoters. *Toxicology and Applied Pharmacology*, **91**, 159-170.
- KLAUNIG, J.E. & RUCH, R.J. (1990). Biology of disease: role of inhibition of intercellular communication in carcinogenesis. *Laboratory Investigation*, **62**, 135-146.
- KLEBER, A.G., RIEGGER, C.B. & JANSE, M.J. (1987). Electrical uncoupling and increase of extracellular resistance after induction of ischemia in isolated, arterially perfused rabbit papillary muscle. *Circulation Research*, **61**, 271-279.
- KNOWLES, W.D., FUNCH, P.G. & SCHWARTZKROIN, P.A. (1982). Electronic and dye coupling in hippocampal CA1 pyramidal cells *in vitro*. *Neuroscience*, **7**, 1713-1722.
- LIN, J.H.-C., WEIGEL, H., COTRINA, M.L., BUENO, E., HANSEN, A.J., HANSEN, T.W., GOLMAN, S. & NEDERGAARD, M. (1998). Gap-junction-mediated propagation and amplification of cell injury. *Nature Neuroscience*, **1**, 494-500.
- LOEWENSTEIN, W.R. (1981). Junctional intercellular communication: the cell-to-cell membrane channel. *Physiological Reviews*, **61**, 829-913.
- LOEWENSTEIN, W.R. & KANNO, Y. (1964). Studies on an epithelial (gland) cell junction. I. Modification of surface membrane permeability. *Journal of Cell Biology*, **22**, 565-586.
- MACVICAR, B.N. & DUREX, F.E. (1982). Electronic coupling between granule cells of rat dentate gyrus: physiological and anatomical evidence. *Journal of Neurophysiology*, **47**, 579-592.
- MATSUMOTO, A., ARAI, Y., URANO, A. & HYODO, S. (1991). Cellular localization of gap junction mRNA in the neonatal rat brain. *Neuroscience Letters*, **124**, 225-228.
- MEGE, R.-M., MATSUZAKI, F., GALLIN, W.J., GOLDBERG, J.I., CUNNINGHAM, B.A. & MUGNANI, E. (1988). Cell junction of astrocytes, ependyma and related cells in the mammalian central nervous system, with emphasis on the hypothesis of a generalized syncytium of supporting cells. In: FEDOROFF, S. and VERNADAKIS, A. (Eds), *Astrocytes*, Vol. 1, pp. 329-371, London, Academic Press.
- O'BEIRNE, M., ANDREW, G.M., BULLOCH, G. & MACVICAR, B.V. (1987). Dye and electronic coupling between cultured hippocampal neurons. *Neuroscience Letters*, **78**, 265-270.
- PAYTON, B.W., BENNETT, M.V.L. & PAPPAS, G.D. (1969). Permeability and structure of junctional membranes at an electronic synapse. *Science*, **166**, 1641-1643.
- PEINADO, A., YUSTE, R. & KATZ, L.C. (1993). Extensive dye coupling between rat neocortical neurons during the period of circuit formation. *Neuron*, **10**, 103-114.
- PERACCHIA, C. (1980). Structural correlates of gap junction permeation. *International Review of Cytology*, **66**, 81-146.
- PERACCHIA, C. & PERACCHIA, L.L. (1980). Gap junction dynamics: reversible effect of hydrogens. *Journal of Cell Biology*, **87**, 719-727.
- PITTS, J.S. & SIMS, J.W. (1977). Permeability of junctions between animal cells. Intercellular transfer of nucleotides but not of macromolecules. *Experimental Cell Research*, **104**, 153-163.
- RAO, G., BARNES, C.A. & MACNAUGHTON, B.L. (1986). Intracellular fluorescent staining with carboxyfluorescein: a rapid and reliable method for quantifying dye-coupling in mammalian central nervous system. *Journal of Neuroscience Methods*, **16**, 251-263.
- RASH, J.E. & YASUMURA, T. (1992). Improved structural detail in freeze-fracture replicas: high-angle shadowing of gap junctions cooled below -170°C and protected by liquid nitrogen-cooled shrouds. *Microscience Research Techniques*, **20**, 187-204.
- REVEL, J.P. & KARNOVSKY, M.J. (1967). Hexagonal array of subunits in intercellular junctions of the mouse ear and liver. *Journal of Cell Biology*, **33**, C7-C12.
- RICHARDS, C.D. & SERCOMBE, R. (1968). Electrical activity observed in guinea-pig olfactory cortex maintained *in vitro*. *Journal of Physiology*, **197**, 667-683.
- RIESKE, E., SCHUBERT, P. & KREUTZBERG, G.W. (1975). Transfer of radioactive material between electrically coupled neurons of the leech central nervous system. *Brain Research*, **84**, 365-382.
- ROBERTSON, J.D. (1963). The occurrence of a subunit pattern in the unit membranes of club endings in Mauthner cell synapses in goldfish brains. *Journal of Cell Biology*, **19**, 201-221.
- ROSE, B. & RUCK, R. (1978). Intracellular pH, intracellular free Ca, and junctional cell-cell coupling. *Journal of Membrane Biology*, **44**, 377-415.
- SCHWARTZKROIN, P.A. (1975). Characteristics of CA1 neurons recorded intracellularly in the hippocampal *in vitro* slice preparation. *Brain Research*, **85**, 423-436.

# Brain Spheroid and Other Organotypic Culture Systems in In Vitro Neurotoxicology

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## Contents

9.1	Introduction
9.2	Brain spheroid culture
9.3	Other organotypic models
9.4	Methodological considerations
9.5	Neurotoxicological investigations
9.6	Recent progress and future directions
9.7	Conclusions
9.8	Acknowledgements
9.9	References

- SHIOSAKA, S., YAMAMOTO, T., HERTZBERG, E.L. & NAGY, J.I. (1989). Gap junction protein in rat hippocampus: correlative light and electron microscope immunohistochemical localization. *Journal of Comparative Neurology*, **281**, 282-297.
- SMITH, J.H., GREEN, N.S., PETERS, S., ROTHERY, S. & SEEVERS, N.J. (1991). Altered patterns of gap junction distribution in ischemic heart disease. An immunohistochemical study of human myocardium using laser scanning confocal microscopy. *American Journal of Pathology*, **139**, 801-821.
- SOCOLAR, S.J. & LOEWENSTEIN, W.R. (1979). Methods for studying transmission through permeable cell-to-cell junctions. In: KORN, D.E. (Ed.) *Methods in Membrane Biology*, Vol. 10, p. 121, New York, Plenum Press.
- SPRAY, D.C., GINZBERG, R.D., MORALES, E.A., GATMAITAIN, Z. & ARIAS, I.M. (1986). Electrophysiological properties of gap junctions between dissociated pairs of rat hepatocytes. *Journal of Cell Biology*, **103**, 135-144.
- STEWART, W.W. (1978). Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell*, **14**, 741-759.
- STRETTON, A.O. & KRAVITZ, E.A. (1968). Neuronal geometry: determination with a technique of intracellular dye injection. *Science*, **162**, 132-134.
- TRAUB, O., DRUGE, P.M. & WILLECKE, K. (1983). Degradation and resynthesis of gap junction protein in plasma membranes of regenerating liver after partial hepatectomy or cholestasis. *Proceedings of National Academy of Sciences USA*, **80**, 755-759.
- TSIEN, R.W. & WEINGART, R. (1976). Inotropic effects of cyclic AMP in calf ventricular muscle studied by a cut end method. *Journal of Physiology (London)*, **260**, 117-141.
- TROSKO, J.E., JONE, C. & CHANG, C.C. (1987). Inhibition of gap junction-mediated intercellular communication *in vitro* by aldrin, dieldrin, and toxaphene: a possible cellular mechanism for their tumor-promoting and neurotoxic effects. *Molecular Toxicology*, **1**, 83-93.
- VAN ZOELN, E.J.J. & TERTOOLEN, L.G.J. (1991). Transforming growth factor-beta enhances the extent of intercellular communication between normal rat kidney cells. *Journal of Biological Chemistry*, **266**, 12075-12081.
- WARNER, A.E., GUTHRIE, S.C. & GILULA, N.B. (1984). Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature*, **311**, 127-131.
- YAMAMOTO, T., SHIOSAKA, S., WHITTAKER, M.E., HERTZBERG, E.L. & NAGY, J.I. (1989). Gap junction protein in rat hippocampus: light microscope immunohistochemical localization. *Journal of Comparative Neurology*, **281**, 269-281.
- YAMAMOTO, C. (1972). Activation of hippocampal neurons by mossy fiber stimulation in thin brain sections *in vitro*. *Experimental Brain Research*, **14**, 423-435.
- YE, Y.X., BOMBICK, D., HIRST, K., ZHANG, G.X., CHANG, C.C., TROSKO, J.E. & BEYER, E.C. (1990). The modulation of gap junctional communication by gossypol in various mammalian cell lines *in vitro*. *Fundamental and Applied Toxicology*, **14**, 817-832.

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- Huang H-JS, Yee J-K, Shew J-Y, et al (1988). Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science*, 242, 1563-1566.
- Knudson AG (1971). Mutations and cancer. Statistical study of retinoblastoma. *Proc Natl Acad Sci, USA*, 68, 820-823.
- Lee W-H, Bookstein R, Hong F, Young L-J, Shew J-Y, Lee EY-HP (1987). Human retinoblastoma susceptibility gene: cloning, identification and sequence. *Science*, 235, 1394-1399.
- Li FP, Fraumeni JF (1982). Prospective study of a family cancer syndrome. *JAMA*, 247, 2692-2694.
- Lothe RA (1992). *Suppressor Genes Involved in Tumorigenesis of Colon and Testis. A Molecular and Cytogenetic Study*. Doctoral thesis, University of Oslo, Norway.
- Mayr E (1982). *The Growth of Biological Thought: Diversity, Evolution, and Inheritance*. Harvard University Press, Cambridge, MA.
- Malkin D, Jolly KW, Barbier Piraux N, et al (1992). Germline mutations of the p53 tumor suppressor gene in children and young adults with second malignant neoplasms. *N Engl J Med*, 20, 1309-1315.
- Seizinger BR, Klinger HP, Junien C, et al (1991). Report of the committee on chromosome and gene loss in human neoplasia. *Cytogenet Cell Genet*, 58, 1080-1096.
- Sideranzky D, Tokino T, Helzlsouer K, et al (1992). Inherited p53 mutations in breast cancer. *Cancer Res*, 52, 2984-2986.
- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science*, 235, 177-182.
- Steeg PS, Choh KH, Leone A (1991). Tumor metastasis and nm23: current concepts. *Cancer Cells*, 3, 257-262.
- Thor AD, Moore DH II, Edgerton SM, et al (1992). Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst*, 84, 845-855.
- Vogelstein B, Fearon ER, Hamilton SR, et al (1988). Genetic alterations during colorectal-tumor development. *N Engl J Med*, 319, 525-532.
- Yunis JJ, Ramsay N (1978). Retinoblastoma and subband deletion of chromosome 13. *Am J Dis Child*, 132, 161-163.

## Chapter 14

# Oncogenes, Tumor Suppressor Genes, and Intercellular Communication in the Oncogeny as Partially Blocked Ontogeny Hypothesis

James E. Trosko, Chia-Cheng Chang, Burra V. Madhukar, and Emmanuel Dupont

*Cancer is a disease of faulty gene expression that results in "partially blocked ontogeny" and an insensitivity to organismic needs.*

V. R. Potter (1988)

## INTRODUCTION

It has often been stated that in understanding cancer, one can understand normal cell growth. Equally important, however, is that in understanding normal cell growth and behavior, insight to the carcinogenic process will be gained. The insights provided by the quotation from Van R. Potter serve to highlight two major principles of higher organism biology that unfortunately, seem to have

been ignored by modern reductionalistic cancer scientists, namely the "hierarchical" (Brody, 1973) and "homeostatic" or "cybernetic" (Potter, 1974; Iversen, 1965) principles.

The normal multicellular organism, starting from a single totipotent stem cell, the fertilized egg, ends up as an organized and orchestrated collection of pluripotent stem, progenitor, and differentiated cells. These cells are capable of contributing to either further growth, development, differentiation, wound healing, or adaptive responses before death ensues. In other words, the human being with all of its functions is not just a collection of 100 trillion individual cells, but an organized and orchestrated collection of interacting groups of different kinds of cells.

These concepts, derived over many years from investigators representing many disciplines (eg, Claude Bernard, W. B. Cannon, P. Weiss, J. L. Kavanagh, O. H. Iversen, E. E. Osgood, and V. R. Potter; see Iversen, 1965; Potter, 1981), postulated the existence of positive and negative regulatory factors that existed between stem/progenitor cells and their differentiated daughters to control growth and differentiation. Clearly, this implied that positive and negative factors altered the target cell's phenotype by some intracellular signaling process. In brief, the conceptual framework postulates that intercellular communication plays a major role in the regulation of cell growth, differentiation, and adaptive response of all the cells of a multicellular organism.

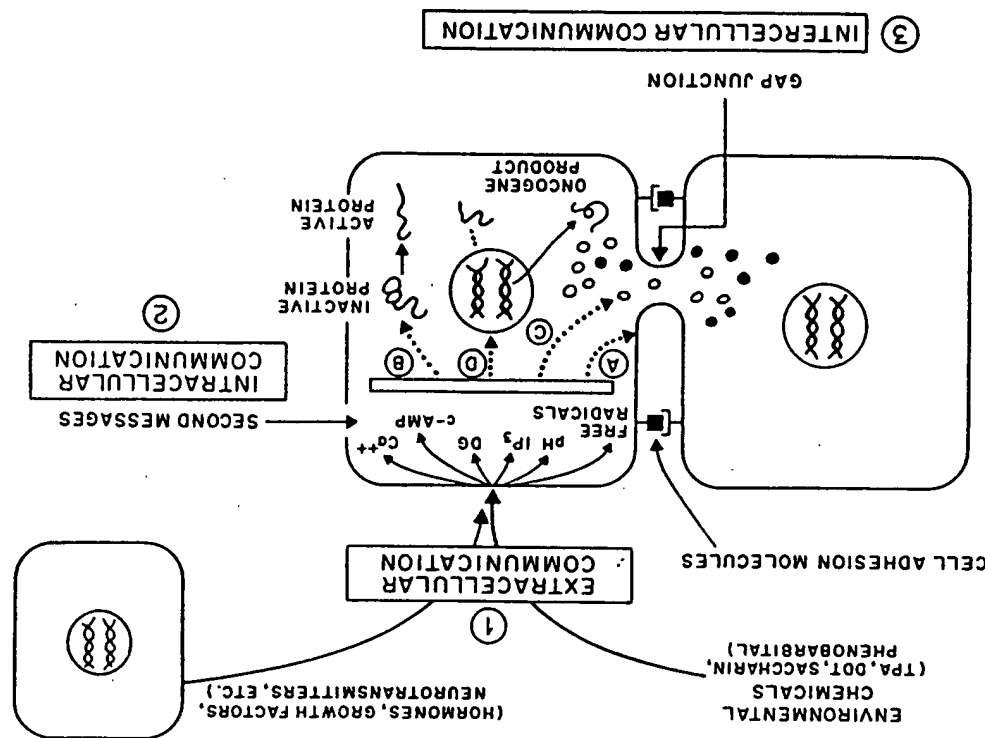
The general pathway for this cybernetic feedback system appears to be via the production of positive factors (Growth factors, hormones, and neurotransmitters, ie, extracellular positive signals) that trigger receptors, and transmembrane signaling elements (intracellular communicating molecules). These signals, in turn, are transferred to neighboring cells or modulate intercellular mechanisms within tissues (Figure 1).

After receiving these signals, the targeted cells alter their physiology in response to these signals, producing negative extracellular signals that feedback to the positive sources. If one accepts this basic concept of all normal higher organisms, then by logic alone one would reason that a blockage or breakdown or any one of these steps (extracellular, intracellular, or intercellular communication) should lead to the inability to control cell growth, differentiation, or adaptive responses of differentiated cells. This would seem to describe the cancer cell.

## THEORIES OF CARCINOGENESIS

All scientific theories must explain observations in order that testable predictions can be made to falsify the theory. The major observations that are to be the focus of this analysis are: (a) normal cells are potentially contact inhibitable; cancer cells appear to be contact-insensitive (Borek & Sachs, 1966); (b) normal cells derived from stem and progenitor cells are capable of terminal differentiation; cancer cells, under their normal growth conditions, do not terminally differentiate (teratomas represent a special case; however, in principle, they do not break this

**Figure 1** Schematic diagram characterizing the postulated link between extracellular and intercellular communication through various intracellular transmembrane signaling mechanisms. Intracellular communication alters membrane function, activates inactive proteins, modulates gap junction function, and modulates gene expression. It provides an integrated view of how the neuroendocrine immune system (mind or brain/body connection) and other multisystem coordinations could occur. While not shown here, activation or altered expression of various oncogenes (antioncogenes) could also contribute to the regulation of gap junction function. Reproduced from Trosko JE, Chang CC (1989). *Toxicology Letters*, 49, 283-295 with permission from Elsevier Science Publishers, Amsterdam, The Netherlands.





rule; Chang et al, 1990); and (c) most, if not all, tumors appear to be of clonal origin (Fialkow, 1979).

Several theories, based on these observations, have been proposed; cancer as a "disease of differentiation" (Markert, 1968; Pierce, 1974); initiation/promotion/progression theory of carcinogenesis (Pitot et al, 1981); and the oncogene/tumor suppressor gene theory of carcinogenesis (Weinberg, 1991). To integrate all of these different but overlapping theories, the theory of cancer as "dysfunctional intercellular communication," first postulated by Loewenstein (1966), was modified to integrate these other theories, as well as to incorporate recent observations related to gap junctional intercellular communication (Trosko et al, 1983; Trosko et al, in press).

In brief, the hypothesis to be developed here is that, starting with a normal stem or progenitor cell, stable alteration of a gene (an oncogene) that controls terminal differentiation but does not alter the control of cell proliferation (ie, contact inhibition or some form of gap junctional intercellular communication) would constitute the initiation phase of carcinogenesis. As long as that initiated stem or progenitor cell is communicating with other normal (heterologous) or initiated (homologous) cells, there will be no cell proliferation. On the other hand, if this gap junctional intercellular communication (GJIC) is inhibited by endogenous (growth factors or hormones) or by exogenous chemicals in a reversible fashion, then clonal expansion of these initiated cells can occur (tumor promotion). Because of their inability to terminally differentiate, these initiated cells, once stimulated to proliferate, will slowly accumulate as a benign monoclonal focus of nonterminally differentiated cells.

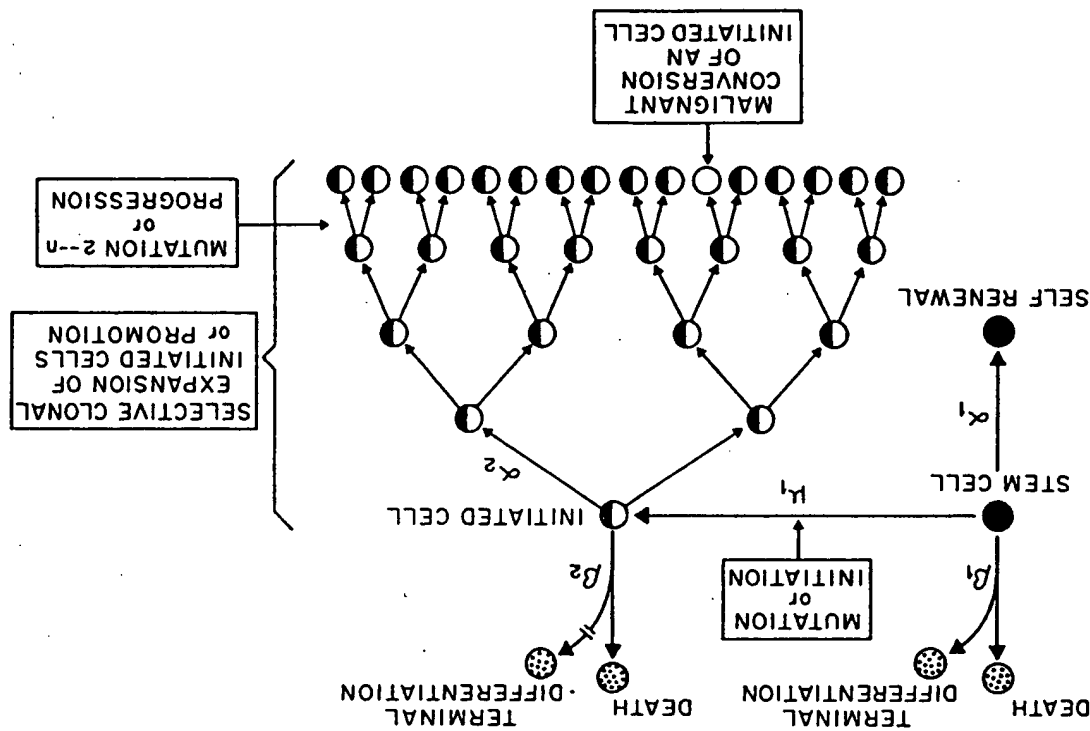
If other irreversible or mutagenic events occur during this process in a gene that stabilizes the blockage of intercellular communication (ie, in another oncogene or tumor suppressor gene), then the progression phase of carcinogenesis could occur (Figure 2).

This hypothesis has now integrated all of the aforementioned theories by means of postulating that a normal cell has the ability to communicate with other normal cells. In other words, initially a normal stem cell, through cell to cell extracellular communication, is regulated by its terminally differentiated daughter. The transition to a normal progenitor involves growth control by means of GJIC. If one of these progenitors is initiated, it will only proliferate, but not terminally differentiate, by either endogenous/exogenous reversible down-regulation of GJIC or by stable down-regulation by imbalance of oncogenes/tumor suppressor gene activities.

#### CELL-CELL COMMUNICATION IN NORMAL GROWTH CONTROL, DIFFERENTIATION, AND ADAPTIVE FUNCTIONS OF DIFFERENTIATED CELLS

The broad concept of homeostasis and cybernetics as it applies to regulatory control of cells during growth, development, differentiation, wound healing, and

Figure 2 The initiation/promotion/progression model of carcinogenesis.  $\beta_1$  = rate of terminal differentiation and death of stem cell;  $\beta_2$  = rate of death, but not of terminal differentiation of the initiated cell ( $- \parallel - \rightarrow$ );  $\alpha_1$  = rate of cell division of stem cells;  $\alpha_2$  = rate of cell division of initiated cells;  $\mu_1$  = rate of the molecular event leading to initiation (ie, possibly mutation);  $\mu_2$  = rate at which second event occurs within an initiated cell. Reproduced from Trosko JE et al (1988). *Gap Junctions*, edited by EL Hertzberg and RG Johnson with permission from Wiley-Liss, New York.



adaptive responses implies extracellular communication from one cell to another over a distance through hormones, growth regulators, neurotransmitters, and extracellular matrices. Intracellular communication includes all the second messages initiated by the extracellular signals. These second messages not only trigger changes intracellularly but can (a) spread to other cells by the intercellular gap junction channels (Saez et al, 1989) or (b) up- or down-regulate the gap junction channels (Spray & Bennett, 1985).

Intercellular communication is mediated by protein channels in membranes of contiguous cells (connexons). Each cell contributes a hemichannel composed of a hexamer of proteins (connexins). Clusters of these connexons allow ions and small molecules (below 1000 daltons) to freely equilibrate between coupled cells. There exists a family of highly conserved genes coding for these proteins (Fishman, Eddy, Shows, et al, 1991). From an evolutionary perspective, the gene for gap junctions appeared when multicellular organisms appeared (Revel, 1988).

Philosophically, the manner by which single-cell organisms (which are devoid of gap junctions) adaptively survived was by means of unlimited growth (contingent only on exogenous nutrients, etc). Multicellular organisms acquired differentiated functions for adaptive means to survive. The transition from unlimited growth to differentiation by a multicellular organism as means to survive coincided with the appearance of the gap junction (Revel, 1988). A multicellular organism needed to have a means to orchestrate the delicate balance of the cells' new roles. Namely, in addition to stimulate cell growth, the organism needed to control cell growth; it had to differentiate; it had to have renewal and wound healing potential; and once terminally differentiated it had to be able to adaptively change from a quiescent state to a functional state. While single cell organisms can communicate through extracellular means and respond to these signals with intracellular second messages, they lacked intercellular communication by means of gap junctions.

Most normal cells of a metazoan (except single mature red blood cells and normal stem cells; Chang et al, 1987; 1990) have gap junctions. These gap junctions appear to play roles of electrotonic synchronization in tissues such as heart or uterine muscle, of metabolic equilibration in nonexcitable tissues, of growth control in contact-inhibited premitotic cells, of differentiation control as in the maturation of sperm and eggs, and of adaptive response as in production of insulin in pancreatic cells (Hertzberg & Johnson, 1988).

When quiescent or contact-inhibited, premitotic cells' gap junctions are up-regulated and functional. When normal cells are stimulated by growth factors (Madhukar et al, 1989; Maldonado et al, 1988), mitogenic tumor promoters (Trosko & Chang, 1988), or wound-healing signals (Radu & Moldovan, 1991), there is a transient decrease or down-regulation of gap junctions. While it is important to determine if gap junctions need to be down-regulated before cell proliferation (cause) or whether the decrease in GJIC is the consequence of cell

proliferation, several recent observations seem to suggest that a decrease in GJIC precedes cell proliferation (Azarnia et al, 1988; Shiba, 1991).

The picture that seems to be emerging is that during the development and adaptive functioning of a mature multicellular organism, cells of the different organs are interacting within and between tissues. Within the tissue, stem cells are interacting with their differentiated daughters (extracellular communication). Progenitor and differentiated cells are communicating by both extra- and intercellular means (de Rooij et al, 1985). In addition, homologous GJIC occurs between some cells (eg, liver epithelial cells couple with like-type cells; hepatocytes couple with hepatocytes; however, liver epithelial cells do not couple with hepatocytes, yet they seem to communicate through extracellular means; Mesnil et al, 1987). Other cells execute GJIC through heterologous means, such as Sertoli cells and spermatogonia (Gilula et al, 1976). Since these extracellular signals are known to modulate GJIC (Madhukar et al, 1989; Maldonado et al, 1988; Larsen, 1983), they could either stimulate cell proliferation (by down-regulation of GJIC in premitotic cells); cell differentiation (by either up- or down-regulation of GJIC in specific homoor heterologous coupling situations); or differentiated functions (Meda et al, 1987).

## DYSFUNCTIONAL GAP JUNCTIONAL INTERCELLULAR COMMUNICATION AND CANCER

If the foregoing discussion of the importance of GJIC is for the regulation of cell growth and differentiation is correct, then a single hypothesis can be postulated.

Postulate I. Normal premitotic cells are characterized by contact inhibition and the ability to terminally differentiate.

Postulate II. Gap junctional intercellular communication is necessary for growth control and terminal differentiation.

Postulate III. Cancer cells are characterized by the loss of growth control and by the inability to terminally differentiate.

Hypothesis. The transition from a normal stem/progenitor cell to a cancer cell must be due partly to its inability to perform GJIC.

Is there evidence to test this hypothesis? First, let it be clear that the hypothesis is based on the concept of gap junctional intercellular communication. In order that cells can perform GJIC: (a) they must be able to adhere (cell adhesion molecules must be functional; Jongen et al, 1991); (b) they must be able to couple their gap junctions; (c) they must be able to transfer ions and small molecules through the gap junctions; and (d) once into cells, these ions

and regulatory molecules must be able to elicit their response. If any of these steps is blocked, GJIC is blocked.

### Most Normal Cells Have GJIC

To date, surveys using either electron micrographic analysis, functional coupling studies, antibodies to connexins, and molecular probes for gap junction messages have shown that most of the normal cells of solid tissues have gap junctions. A few cell types do not, such as normal stem cells (Chang et al, 1987; 1990) or mature red blood cells (although they appear in the early maturation process; Dainiak, 1991; Rosendaal et al, 1991).

### Most, If Not All, Cancer Cells Have Dysfunctional GJIC

Clearly, not all cancer cells have been examined. However, very early in the study of gap junctions, Loewenstein (1966) noticed a paucity of GJIC in cancer cells compared to normal cells. While exceptions have been noted (Larsen, 1983), because of the nature of the studies these exceptions do not constitute rigorous negation of the hypothesis. For example, detection of morphological presence of gap junctions does not prove they are functional; some cancer cells do communicate homologously, but not heterologously (Yamasaki et al, 1987); some cancer cells may transfer ions used to measure GJIC, but may not have the cellular signal to suppress growth, etc. Moreover, many studies have indicated a clear decrease or down-regulation of GJIC in various cancer cells (Kanno, 1985).

### Most, If Not All, Endogenous and Exogenous Tumor-Promoting Chemicals Reversibly Inhibit GJIC

In the experimental protocol of initiation, promotion, and progression of carcinogenesis in animals, the promotion phase, operationally, is a potentially interruptable or reversible step. In *in vitro* and *in vivo* experiments with endogenous hormones, growth factors and exogenous tumor-promoting chemicals (ie, 12-O-tetradecanoylphorbol-13-acetate, TPA; polybrominated biphenyls, PBBs; phenobarbital, etc; Trosko & Chang, 1988), gap junctional communication can be reversibly down-regulated, either at the posttranslational levels (eg, phosphorylation of GJ protein) or transcriptional level (Bennett et al, 1991). One known tumor promoter, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was claimed to be a noninhibitor of GJIC (Lincoln et al, 1987). However, it has not been demonstrated that TCDD biologically affected those cells or if those cells had receptors for TCDD. Even physical promotion, such as tissue necrosis, cytotox-

icity, or wounding, has been associated with the down-regulation of gap junctions (Yancey et al, 1979; Traub et al, 1983).

### Certain Oncogenes Down-Regulate GJIC in a Dose-Dependent Fashion

If tumor-promoting chemicals, which act as growth factors for initiated cells, and growth factors, which can be tumor promoters, inhibit GJIC, then oncogenes, which code for growth factors, growth factor receptors, or transmembrane mitogenic signaling elements, should be able to down-regulate GJIC either transiently (proto-oncogenes) or stably (activated oncogenes) when they transform cells. To date, a series of transforming activated oncogenes, eg, *src*, *ras*, *neu*, *raf*, *mos*, have been shown to down-regulate GJIC in various cell types and in a dose-dependent fashion (Azarnia et al, 1988; Atkinson et al, 1981; Atkinson et al, 1986; Azarnia & Loewenstein, 1984; Azarnia & Loewenstein, 1987; Chang et al, 1985; Dotto et al, 1989; El-Fouly et al, 1989; Bignami et al, 1988; El-Fouly et al, 1988; Kalimi et al, 1992). In several cell systems, eg, rat liver epithelial oval cells, the oncogenes *ras*, *raf*, and *neu* significantly reduced GJIC; however, together with an expressed *myc*, GJIC was reduced even more (Kalimi et al, *in press*). In addition, the oncogene *ras*, when either linked to a metallothionein promoter or exposed to TPA, down-regulated communication either in a direct relationship to the p21 protein produced or synergized with TPA to activate protein kinase-C to down-regulate GJIC completely (Oh et al, 1991; Brissette et al, 1991; de Feijter et al, 1992).

### Several Tumor Suppressor Genes Have Been Shown to Up-Regulate GJIC

If the current paradigm is correct that a balance (yin-yang) between oncogenes and tumor suppressor genes exists in normal, GJIC cells, then one can surmise that in a nontransformed cell that has an activated oncogene in it a tumor suppressor gene must have negated or ameliorated its effect on the down-regulation of gap junctions.

Lee et al (1991) and Kalimi et al (1990) have recently shown that two potential tumor suppressor genes appear to be correlated with the up-regulation of GJIC, or the prevention of a down-regulation of GJIC. Since tumor-promoting chemicals, activated oncogenes, and growth factors that down-regulate GJIC do so through many biochemical mechanisms (Spray et al, 1988), one can imagine there will be several ways to ameliorate or prevent such down-regulation. For example, if TPA and *ras* affect GJIC by their ability to phosphorylate the GJ protein through protein kinase-C, then dephosphorylation activated by tumor suppressor genes might be one mechanism through which a particular tumor suppressor gene could act. Other examples are in the next two sections.

### Anti-Tumor Promoters Such as Retinoids and C-AMP Can Up-Regulate GJIC

Again using the experimental protocol of initiation/promotion/progression in mouse skin, it has been shown that various retinoids could block the tumor-promoting action of TPA (Verma et al, 1979). If the cellular mechanism by which TPA acts as a tumor promoter is via its ability to down-regulate GJIC, then one would predict inhibition of GJIC by TPA should be prevented or ameliorated by the action of retinoids. The results of experiments in an *in vitro* transformation system appear to be consistent with the prediction, in that retinoids prevented the TPA reduction of GJIC (Mehta et al, 1989; Mehta & Loewenstein, 1991).

Recently, Rivedal and Sanner (1992) showed that retinoids, which can promote transformation in Syrian hamster embryo cells in contrast with its ability to inhibit the induction of transformation of C3H10T1/2 and BALB/C 3T3, down-regulate GJIC. In all three cell types, enhancement of communication by retinoids was related to reduced transformation, whereas inhibition of communication was related to enhanced induction of transformation.

Several *in vitro*, as well as *in vivo*, experiments have also shown that increased levels of intracellular C-AMP are associated with enhanced GJIC (Flagg-Newton et al, 1981; Demazure & Scheuerman, 1985). In initiation/promotion/progression experiments one would then predict increases in C-AMP ought to prevent the promotion of tumors.

### Antioncogenic Drugs Such as Lovastatin Reverse RAS-Down-Regulation of GJIC

As understanding of how each oncogene acts to down-regulate GJIC grows, it might be possible to interfere with its mechanism of action. One oncogene, the *H-ras* appears to be functional only after the p21 oncoprotein is bound to the under surface of the plasma membrane. It has been shown that lovastatin can prevent that process (Casey et al, 1989). Interestingly, it also prevents the growth of *ras*-transformed cells in nude mice (Sebti et al, 1991). Recently, it has been shown that lovastatin prevents the *ras*-down-regulation of GJIC in a reversible fashion (Ruch et al, 1993). If a cell has genes that perform the same function in *H-ras* expressed cells, these genes would be considered tumor suppressor genes.

### Gap Junction Gene Transfection of GJIC-Deficient and Tumorigenic Cells Restores Their Ability to Communicate

Most assuredly, the ultimate test of the hypothesis would be the restoration of GJ expression in a GJIC-deficient and tumorigenic cell and the subsequent res-

oration of GJIC and reduction of tumorigenicity. Several recent attempts have been successful in the restoration of GJIC in noncommunication tumorigenic cells (Eghbali et al, 1991; Fishman et al, 1991; Mehta et al, 1991; Zhu et al, 1991). Whether these recommunicating tumor cells retain their tumorigenic properties awaits further experimentation.

### Gap Junction Antisense Gene Transfected GJIC-Proficient Normal Rat Liver Cells Decreases GJIC

The complementary experiment to the one described above that should test the hypothesis is to transfect an expressible gap junction antisense gene into a GJIC-proficient and normal cell to convert it to a noncommunicating cell and to determine if it is tumorigenic. The authors are currently attempting to express the antisense connexin 43 gap junction gene in normal, GJIC-proficient rat liver epithelial cells. These cells when placed in the nude mice would be predicted to give rise to tumors.

### GJIC-Deficient Mutants Derived from Nontumorigenic Rat Liver Cells are Tumorigenic

Several GJIC-deficient mutants have been isolated from the immortal but nontumorigenic GJIC-proficient rat liver WB cell line (Tsao et al, 1984). These mutants have been shown to be defective in posttranslational phosphorylation of the C43 gap junction protein (Oh et al, *in press*). One of these mutants, *aB-1*, has been inoculated into rat livers through portal vein injection for tumorigenicity tests. Liver tumors developed in 10 of 12 rats injected with *aB-1* cells in 10 weeks. The results were repeated in two different laboratories (J. Klaunig of the University of Indiana and C. Welsch of Michigan State University). Compared to *neu* oncogene transfectants that developed liver tumors in three weeks, it took a longer time for the *aB-1* cells to develop the tumors, indicating that GJIC deficiency by itself might be necessary, but insufficient, for triggering the mitogenic process leading to the clonal expansion of tumorigenic transformants in a cell population. In other words, chemical promoters, oncogenes, or growth factors, which act as mitogens for initiated cells, probably can trigger, in a coordinate way, both the down-regulation of GJIC (needed to inhibit contact inhibition) and the onset of the DNA synthesis/mitogenic process. A mutation in a gene that affects only the gap junction structure or function, in and of itself, would not be sufficient to initiate the mitogenic synthetic mechanisms.

### SUMMARY

Given that a multicellular organism must regulate its functions of cell proliferation, differentiation, wound healing, and adaptive responses through a homeos-

tatic-cybernetic system, a hypothesis has been advanced, based on the assumption that an integrated extra-, intra- and intercellular communication system exists within and between cells of different tissues, that stable interference of gap junctional intercellular communication, which could occur in a single initiated (nonterminally differentiated) stem or progenitor cell, would lead to cancer. Several tests of the theory were reviewed and all, with varying degrees of rigor, seem to be consistent with the hypothesis. Since gap junctional intercellular communication exists in all organs and tissues, and since it plays a role not only in the regulation of cell proliferation of premitotic cells, eg, its potential role in cancer, blockage of GJIC probably plays roles in many other disease states, for example, teratogenesis (Trosko et al, 1982; Warner et al, 1984); neurotoxicity (Naus et al, 1991; Trosko et al, 1987); reproductive dysfunction (Ye et al, 1990); cardiovascular diseases (Radu & Moldovan, 1991; Smith et al, 1991); cataract formation (Tanaka et al, 1990); and cholestasis (Traub et al, 1983).

In the words of Theodosius Dobzhansky (1973), "Nothing in biology makes sense except in the light of evolution" (p. 125). The gap junction, having shown up in the evolution of metazoans and having been associated with the regulation of cell proliferation and differentiation, has been highly conserved. Any genetic alteration affecting its normal function would block its ability to regulate growth and differentiation. Cancer seems to be the result of such alterations in the somatic tissue, which has been biologically prohibited in the germline since a dysfunctional gap junction expressed after conception would, no doubt, be a lethal event.

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## REFERENCES

- Atkinson MM, Anderson SK, Sheridan JD (1986). Modification of gap junctions in cells transformed by a temperature-sensitive mutant of Rous sarcoma virus. *J Membr Biol*, 91, 53-64.
- Atkinson MM, Menko AS, Johnson RG, Sheppard IR, Sheridan JD (1981). Rapid and reversible reduction of junctional permeability in cells infected with a temperature sensitive mutant of avian sarcoma virus. *J Cell Biol*, 9, 573-578.
- Azamia R, Loewenstein WR (1984). Intercellular communication and the control of growth: alteration of junctional permeability by the *src* gene. A study with temperature-sensitive mutant Rous sarcoma virus. *J Membr Biol*, 82, 191-205.
- Azamia R, Loewenstein WR (1987). Polymavirus middle T antigen middle T antigen down regulates junctional cell to cell communication. *Mol Cell Biol*, 7, 946-950.
- Azamia R, Reddy S, Kimiecki TE, Shalloway D, Loewenstein WR (1988). The cellular *src* gene products regulates junctional cell to cell communication. *Science*, 239, 398-400.
- Bennett MVL, Barrio LC, Bargiello TA, Spray DC, Hertzberg E, Saez JC (1991). Gap junctions: new tools, new answers, new questions. *Neuron*, 6, 305-320.
- Bignami M, Rosa S, Fulcone G, Tuto F, Katoh F, Yamasaki H (1988). Specific viral oncogenes cause differentiated effects on cell to cell communication relevant to the suppression of the transformed phenotype by normal cells. *Mol Carcinogen*, 1, 67-75.
- Borek C, Sachs L (1966). The difference in contact inhibition of cell replication between normal cells and cells transformed by different carcinogens. *Proc Natl Acad Sci USA*, 56, 1705-1711.
- Brisette JL, Kumar NM, Gilula NB, Dotto GP (1991). The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the *ras* oncogene modulate expression and phosphorylation of gap junction proteins. *Mol Cell Biol*, 11, 5364-5371.
- Brody H (1973). A systems view of man: implications for medicine, science and ethics. *Perspect Biol Med*, 17, 71.
- Casey PJ, Soliski PA, Der CJ, Buss JE (1989). p21ras is modified by a farnesyl isoprenoid. *Proc Natl Acad Sci USA*, 86, 8323-8327.
- Chang CC, Nakatsuka S, Kalimi G, Trosko JE, Welsch CW (1990). Characterization of two types of normal human breast epithelial cells that are either deficient or proficient in gap junctional intercellular communication. *J Cell Biochem Suppl*, 14B, 331.
- Chang CC, Trosko JE, El-Fouly MH, Gibson-D'Ambrosio RE, D'Ambrosio SM (1987). Contact insensitivity of a subpopulation of normal human fetal kidney epithelial cells and of human carcinoma cell lines. *Cancer Res*, 47, 1634-1645.
- Chang CC, Trosko JE, Kung HJ, Rombick D, Matsumura F (1985). Potential role of the *src* gene product in inhibition of gap junctional communication in NIH 3T3 cells. *Proc Natl Acad Sci USA*, 82, 5360-5384.
- Dainiak N (1991). Surface membrane-associated regulation of cell assembly, differentiation, and growth. *Blood*, 78, 264-276.
- de Feijter AW, Trosko JE, Krizman DB, Lebovitz RM, Lieberman MW (1992). Increased levels of HrasT24 protein correlate with extent of loss of gap junction function in rat liver epithelial cells. *Mol Carcinog*, 5, 205-212.
- Demaziere AMGL, Scheuerman DW (1985). Increased gap junctional area in the rat liver after administration of dibutyl cAMP. *Cell Tissue Res*, 239, 651-655.
- de Rooij DG, Lok D, Weenk D (1985). Feedback regulation of the proliferation of the undifferentiated spermatogonia in the Chinese hamster by the differentiating spermatogonia. *Cell Tissue Kinet*, 18, 71-81.
- Dobzhansky T (1973). Nothing in biology makes sense except in the light of evolution. *Amer Biol*, 35, 125-129.
- Dotto GP, El-Fouly MH, Nelson C, Trosko JE (1989). Similar and synergistic inhibition of gap-junctional communication by *ras* transformation and tumor promoter treatment of mouse primary keratinocytes. *Oncogene*, 4, 637-641.
- Eghbali B, Kessler JA, Reid LM, Roy C, Spray DC (1991). Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth in vivo. *Proc Natl Acad Sci USA*, 88, 10701-10705.

- El-Fouly MH, Trosko JE, Chang CC (1988). Phenotypic transformation and inhibition of gap-junctional intercellular communication in epithelial and mesenchymal cells by the *neu* oncogene. 4th Annual Oncogene Meeting, *Frederick, MD*, July 5-9, 1988. (Abstract)
- El-Fouly MH, Trosko JE, Chang CC, Warren ST (1989). Potential role of the human *Ha-ras* oncogene in the inhibition of gap junctional intercellular communication. *Mol Carcinogen*, 2, 131-135.
- Fialkow PJ (1979). Clonal origin of human tumors. *Am Rev Med*, 30, 135-176.
- Fishman GI, Eddy RL, Shows TB, Rosenthal L, Leinwand LA (1991). The human connexin gene family of gap junction proteins: distinct chromosomal locations but similar structures. *Genomics*, 10, 250-256.
- Fishman GI, Moreno AP, Spray DC, Leinwand LA (1991). Functional analysis of human cardiac gap junction channel mutants. *Proc Natl Acad Sci USA*, 88, 3525-3529.
- Flagg-Newton JL, Dahl G, Loewenstein WR (1981). Cell junction and cyclic AMP: upregulation of junctional membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. *J Membr Biol*, 63, 105-121.
- Gilula NB, Faucett DW, Aoki A (1976). The Sertoli cell occluding junctions and gap junctions in mature and developing mammalian testis. *Develop Biol*, 50, 142-168.
- Hertzberg EL, Johnson RG (1988). *Gap Junctions*. Alan R Liss, Inc, New York.
- Iversen OH (1965). Cybematic aspects of the cancer problem. In *Progress in Biocybernetics*, edited by N Wiener and JP Schade, Elsevier Publishing Co, Amsterdam, pp. 76-110.
- Jongen WMF, Fitzgerald DJ, Asamoto M, et al (1991). Regulation of connexin 43-mediated gap junctional intercellular communication by CA2+ in mouse epidermal cells is controlled by E-cadherin. *J Cell Biol*, 114, 545-555.
- Kalimi G, Chang CC, Edwards P et al (1990). Re-establishment of gap junctional communication in a nontumorigenic Hela-normal human fibroblast hybrid. *Proc Am Assoc Cancer Res*, 31, 318.
- Kalimi GH, Trosko JE, Hampton LL, Thorgeirsson SS, Huggett AC (1992). Gap junctional intercellular communication in *raf* and *ras* transformed rat liver epithelial cell lines. *Mol Carcinogen*, 5, 301-310.
- Kanno Y (1985). Modulation of cell communication and carcinogenesis. *Jpn J Physiol*, 35, 693-707.
- Larsen WJ (1983). Biological implications of gap junction structure, distribution and composition: a review. *Tissue Cell*, 15, 645-671.
- Lee SW, Tomasetto C, Sager R (1991). Positive selection of candidate tumor-suppressor genes by subtractive hybridization. *Proc Natl Acad Sci USA*, 88, 2825-2829.
- Lincoln DW, Kampck SJ, Gierthy JF (1987). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) does not inhibit intercellular communication in Chinese hamster V79 cells. *Carcinogenesis*, 8, 1817-1820.
- Loewenstein WR (1966). Permeability of membrane junctions. *Ann NY Acad Sci*, 137, 441-472.
- Madhukar BV, Oh SY, Chang CC, Wade M, Trosko JE (1989). Altered regulation of intercellular communication by epidermal growth factor, transforming growth factor-beta and peptide hormones in normal human keratinocytes. *Carcinogenesis*, 10, 13-20.
- Maldonado PE, Rose B, Loewenstein WR (1988). Growth factors modulate junctional cell-to-cell communication. *J Membr Biol*, 106, 203-210.
- Markert C (1968). Neoplasia: a disease of cell differentiation. *Cancer Res*, 28, 1908-1914.
- Meda P, Bruzzone R, Chanson M, Bosco D, Orci L (1987). Gap junctional coupling modulates secretion of exocrine pancreas. *Proc Natl Acad Sci USA*, 84, 4901.
- Mehta P, Bertram JS, Loewenstein WR (1989). The actions of retinoids on cellular growth correlate with their actions on gap junctional communication. *J Cell Biol*, 108, 1053-1065.
- Mehta PP, Hotz-Wagenblatt A, Rose B, Shalloway P, Loewenstein WR (1991). Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. *J Membr Biol*, 124, 207-225.
- Mehta PP, Loewenstein WR (1991). Differential regulation of communication by retinoic acid in homologous and heterologous junctions between normal and transformed cells. *J Cell Biol*, 113, 371-379.
- Mesnil M, Frasin JM, Piccoli C, Yamasaki H, Gugen-Guillouzo C (1987). Cell contact but not junctional communication (dye coupling) with biliary epithelial cells is required for hepatocytes to maintain differentiated functions. *Exp Cell Res*, 173, 524-533.
- Naus CCG, Bechberger JF, Paul DL (1991). Gap junction gene expression in human seizure disorder. *Exp Neurol*, 111, 198-203.
- Oh SY, Grupe CG, Murray AW (1991). Phorbol ester induces phosphorylation and down-regulation of connexin 43 in WB cells. *Biochim Biophys Acta*, 1094, 243-245.
- Oh SY, Madhukar BV, Chang CC, Trosko JE, Beyer E (in press). Characterization of gap junctional communication deficient mutants from a hypoxanthine guanine phosphoribosyl transferase rat liver epithelial cell line. *Eur J Cell Biol*
- Pierce GP (1974). Neoplasms, differentiation and mutations. *Am J Pathol*, 77, 103-118.
- Pitot HC, Goldworthy T, Moran S (1981). The natural history of carcinogenesis: implications of experimental carcinogenesis in the genesis of human cancer. *J Supramol Struct Cell Biochem*, 17, 133-146.
- Potter VR (1974). Probabilistic aspects of the human cybernetic machine. *Perspect Biol Med*, 17, 164.
- Potter VR (1981). The present status of the blocked ontogeny hypothesis of neoplasia: the thalassemia connection. *Oncodevelop Biol Med*, 2, 243-266.
- Potter VR (1988). On the road to the block ontogeny theory. *Adv Oncol*, 4, 3-8.
- Radu A, Moldovan N (1991). 4-Hydroxynonenol reduces junctional communication between endothelial cells in culture. *Exp Cell Res*, 196, 121-126.
- Revel JP (1988). The oldest multicellular animal and its junction. In *Gap Junctions*, edited by EL Hertzberg and RG Johnston. Alan R Liss, Inc., New York, pp. 135-149.
- Rivedal E, Sanner T (1992). Regulation of gap junctional communication in Syrian hamster embryo cell by retinoic acid and 12-O-tetradecanoyl-phorbol-13-acetate. *Carcinogenesis*, 13, 199-203.

- Rosendaal M, Grogan A, Green CR (1991). Direct cell-cell communication in the blood-forming system. *Tissue Cell*, 23, 457-470.
- Ruch RJ, Madhukar BV, Trosko JE, Kluinig JE (1993). Reversal of *ras*-induced inhibition of gap-junctional intercellular communication, transformation, and tumorigenesis by Lovastatin. *Mol Carcinogen*, 7, 50-59.
- Saez JC, Connor JA, Spray DC, Bennett MVL (1989). Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate and to calcium ions. *Proc Natl Acad Sci USA*, 86, 2708-2712.
- Sebit SM, Tkalecic GT, Jani JP (1991). Lovastatin, a cholesterol biosynthesis inhibitor, inhibits the growth of human H-ras oncogene transformed cells in nude mice. *Cancer Commun*, 3, 141-147.
- Shiba Y (1991). Regulation of gap junctional intercellular communication and control of cell growth. *Jpn J Cell Biol*, 44, 13(a).
- Smith JH, Green CR, Peters NS, Rothery S, Severs NJ (1991). Altered patterns of gap junction distribution in ischemic heart disease—an immunohistochemical study of human myocardium using laser scanning confocal microscopy. *Ann J Pathol*, 139, 801-821.
- Spray DC, Bennett MVL (1985). Physiology and pharmacology of gap junctions. *Ann Rev Physiol*, 47, 281-303.
- Spray DC, Saez JC, Burt JM et al (1988). Gap junctional conductance: multiple sites of regulation. In *Gap Junctions*, edited by EL Hertzberg and RG Johnson. Alan R Liss, Inc, New York, pp. 227-244.
- Tanaka T, Sakai M, Fujimoto K, Ogawa K (1990). Morphometric analysis of gap junctions in the rat lens during cataract formation. *Acta Histochem Cytochem*, 23, 781-792.
- Traub O, Druge PM, Willecke K (1983). Degradation and resynthesis of gap junction protein in plasma membranes of regenerating liver after partial hepatectomy or cholestasis. *Proc Natl Acad Sci USA*, 80, 733-739.
- Trosko JE, Chang CC (1988). Nongenotoxic mechanisms in carcinogenesis: role of inhibited intercellular communication. In *Banbury Report 31: Carcinogen Risk Assessment: New Directions in the Qualitative and Quantitative Aspects*, edited by RW Hart and FG Hoerger. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, pp. 139-170.
- Trosko JE, Chang CC (1989). Stem cell theory of carcinogenesis. *Toxicol Lett*, 49, 283-295.
- Trosko JE, Chang CC, Madhukar BV, Dupont E (in press). Intercellular communication: a paradigm for the interpretation of the initiation/promotion/progression model of carcinogenesis. In *Chemical Induction of Cancer: Modulation and Combination Effects*, edited by JC Arcos. Academic Press, New York.
- Trosko JE, Chang CC, Madhukar BV, Oh SY, Bombick D, El-Fouly MH (1988). Modulation of gap junctional intercellular communication by tumor promoting chemicals, oncogenes, and growth factor during carcinogenesis. In *Modern Cell Biology, Vol. 7: Gap Junctions*, edited by EL Hertzberg and RG Johnson, Alan R. Liss, New York, pp. 435-448.
- Trosko JE, Chang CC, Medcalf A (1983). Mechanisms of tumor promotion: potential role of intercellular communication. *Cancer Invest*, 1, 511-526.
- Trosko JE, Chang CC, Netzloff M (1982). The role of inhibited cell-cell communication in teratogenesis. *Teratogenesis Carcinogenesis Mutagen*, 2, 31-45.

- Trosko JE, Jone C, Chang CC (1987). Inhibition of gap junctional-mediated intercellular communication in vitro by Aldrin, Dieldrin, and Toxaphene: a possible cellular mechanism for their tumor-promoting and neurotoxic effects. *Mol Toxicol*, 1, 83-93.
- Tsao MS, Smith JD, Nelson KG, Grisham JW (1984). A diploid epithelial cell line from normal adult rat liver with phenotypic properties of oval cells. *Exp Cell Res*, 154, 38-52.
- Verma AK, Shapas BG, Rice HM, Boutwell RK (1979). Correlation of the inhibition by retinoids of tumor promoter-induced mouse epidermal ornithine decarboxylase activity and of skin tumor promotion. *Cancer Res*, 39, 419-425.
- Warner AE, Guthrie SC, Gilula NB (1984). Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature*, 311, 127-131.
- Weinberg RA (1991). Tumor suppressor genes. *Science*, 254, 1138-1146.
- Yamasaki H, Hollstein M, Mesnil M, Martel N, Aguelon AM (1987). Selective lack of intercellular communication between transformed and nontransformed cells as a common property of chemical and oncogene transformation of BALB/c3T3 cells. *Cancer Res*, 47, 5658-5664.
- Yancey SB, Easter D, Revel JP (1979). Cytological changes in gap junctions during liver regeneration. *J Ultrastruct Res*, 67, 229-242.
- Ye YX, Bombick D, Hirst K, Zhang GX, Chang CC, Trosko JE, Akera T (1990). The modulation of gap junctional communication by gossypol in various mammalian cell lines in vitro. *Fundam Appl Toxicol*, 14, 817-832.
- Zhu D, Caveney S, Kidder GM, Naus CCG (1991). Transfection of C6 glioma cells with connexin 43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. *Proc Natl Acad Sci USA*, 88, 1883-1887.

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# DERMATOTOXICOLOGY

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*Fifth Edition*

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## Chapter Twelve

### MOLECULAR BASIS OF ALLERGIC CONTACT DERMATITIS

*Jean-Pierre Lepoittevin and Valérie Berl*

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#### INTRODUCTION

Among the pathological conditions in which chemistry plays an especially important role is, without doubt, contact allergy. Chemical reactions and interactions are involved throughout the biological process that will result in the patient developing delayed hypersensitivity, whether it be during the crossing of the cutaneous barrier (mainly controlled by the physicochemical properties of the allergen), during the formation of the hapten-protein complex (in which chemical bonds are involved), or during the phenomena of recognition between the antigen and the receptors on T lymphocytes (involving a discipline undergoing rapid development, that of supramolecular chemistry).

Recently, there has been a major step forward in our understanding of the molecular basis of hapten recognition by T cells. Nevertheless, this does not eliminate the need to understand the characteristics of the preceding processes, as it is true to say that the properties of a chemical are implicit in its molecular structure.

To cause sensitization, a compound has to penetrate the skin (Potts and Guy, 1992), where it may be metabolized (Hotchkiss, 1992), and react with Langerhans cell surface proteins to form new chemical structures that are recognized as foreign. We discuss in this chapter the way low-molecular-weight chemicals react with skin proteins to form complete antigens and how these structures are recognized by T-cell receptors.

#### SOME CHEMICAL REMINDERS

Haptens (small molecules with a molecular mass less than 1000 Da) interact with biological macromolecules by mechanisms leading to the formation of bonds of various strengths between the two entities. These bonds, known as chemical bonds, are the result of electronic interactions between atoms and are characterized by the energy that they bring into play, a reflection of their stability. This energy is what must be provided to break the bond between the two atoms. In general, a distinction is made between weak interactions, involving energy levels from a few calories to around 12 kcal/mol of complex, and strong interactions, covalent or coordinate bonds, with bond energies ranging from 50 to 100 kcal/mol.

#### Weak Interactions

Weak interactions are normally grouped into three main categories: hydrophobic bonds, dipolar bonds, and certain ionic bonds. Although these weak interactions involve modest energy levels

and produce complexes of low stability, they are nonetheless of great biological importance, as they control virtually all the phenomena of recognition between receptors and substrates.

Hydrophobic bonds represent the capacity of organic molecules to organize themselves in water so as to minimize the contact area that they expose to the aqueous solvent. It is by such means that hydrophobic molecules insert themselves into the phospholipid bilayers of cell membranes and into the hydrophobic regions of proteins or membrane receptors. These hydrophobic bonds, which involve energies of the order of 10–20 cal/A<sup>2</sup>/mol, seem, nevertheless, to play an important role in allergies to very lipophilic products (Darley et al., 1977), such as the allergens from poison ivy (*Rhus radicans* L.) or poison oak (*Rhus diversiloba* T.). This could also be of importance for the interactions of haptens with the lipophilic domains of antigen-presenting cells.

Dipolar bonds are electrostatic interactions between preexisting or induced dipoles. In actual fact, the electron clouds do not always have a uniform charge density (these variations result from the structure of the molecule), and the zones of high electron density can interact electrostatically with zones of low electron density (permanent dipoles). Electron clouds can also be deformed and polarized as they approach one another, thus creating induced dipoles. The interaction between these dipoles is known as van der Waals bonding, with energies of the order of 50–500 cal/mol. Hydrogen bonds are a special case of dipolar interaction. They occur between a hydrogen atom, bound to an electron-attracting atom, and an electron-rich atom. The energy of these bonds can be as high as 5 kcal/mol.

Ionic bonds are electrostatic interactions between preexisting and generally localized charges on organic molecules or minerals. Such interactions occur, for example, between the charged amino acids in proteins and are therefore important in recognition phenomena.

### Strong Interactions

Strong interactions, mainly covalent bonds, result when two atoms share a pair of electrons, and are classically represented in chemical formulas by dashes. They involve energies of the order of 50–100 kcal/mol and are therefore very stable compared with the weak interactions. The two electrons required for bond formation can be contributed by both partners, which is called a radical reaction, or can be provided by one of the atoms, which is especially electron rich, and shared with the electron-poor atom; this case is referred to as a reaction between a nucleophile (electron rich) and an electrophile (electron poor). These two terms, nucleophile and electrophile, represent the capacity of a molecule, or rather an atom of this molecule, to donate or accept electrons to form a bond. Nucleophilic centers are rich in electrons and are therefore partially negatively charged, while electrophilic centers, deficient in electrons, are partially positively charged.

### Mechanisms of Bond Formation

The main mechanisms for the formation of covalent bonds involved in contact allergy can be grouped into three main categories: nucleophilic substitutions, on either a saturated or unsaturated center, and nucleophilic additions.

Nucleophilic substitution on a saturated center (Figure 1) involves the attack by an electron-rich nucleophile on an electron-poor electrophilic center. As the electrophilic carbon already has four single bonds, a new bond can only be formed if one of the existing bonds is broken. The overall effect will therefore be a substitution of one of the groups (the leaving group) by the nucleophile.

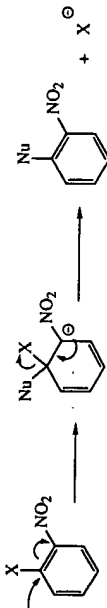
A nucleophilic substitution reaction can also take place at an unsaturated center (a carbon with one or more multiple bonds). In this case, although the overall result is again a substitution, the mechanism is slightly different. The presence of a multiple bond allows the formation of a saturated intermediate and the subsequent reformation of the multiple bond, permitted by the departure of the leaving group, resulting in the substitution product. This mechanism is

### Molecular Basis of ACD

#### - Nucleophilic substitution on a saturated center



#### - Nucleophilic substitution on an unsaturated center



#### - Nucleophilic addition

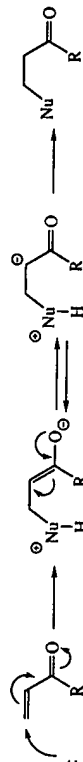


Figure 1. Principal mechanisms of covalent bond formation seen in contact allergy.

illustrated in the aromatic series in which it is all the more favored by attracting groups (e.g. nitro), which stabilize the intermediate.

Nucleophilic addition is simply the addition (with no leaving group) of a nucleophilic atom to an unsaturated electrophilic center (containing one or more multiple bonds). This mechanism is very similar to the first stage of nucleophilic substitution on an unsaturated center, but the absence of a leaving group rules out the reformation of the multiple bond. A saturated compound is thus produced.

### Coordination Bonds

Another type of relatively strong bond, comparable to covalent bonds, is found; this occurs between metals or metal salts and electron-rich atoms (mainly heteroatoms, such as nitrogen or oxygen). These interactions, known as coordinate bonds, permit these electron-rich groups (the ligands) to transfer part of their electron density to the metal and increase its stability. Coordinate bonds are characterized by the number of ligands and by a geometry characteristic both of the metal and of its degree of oxidation (Figure 2). For example, cobalt(II) (Co<sup>2+</sup>) is

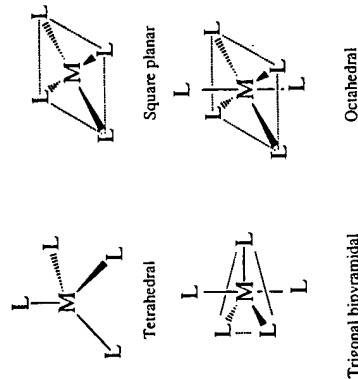


Figure 2. Examples of coordination geometry.

characterized by a tetrahedral arrangement, nickel(II) ( $Ni^{2+}$ ) by a square planar tetra coordinated arrangement, and chromium(III) ( $Cr^{3+}$ ) by a six-ligand octahedral arrangement. The number of ligands and the geometry of these coordination complexes determine whether the metals are allergenic and control cross-reactions.

### PRINCIPAL ELECTROPHILIC CHEMICAL GROUPS PRESENT IN CONTACT ALLERGENS

Many chemical groups have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. Table 1 shows those chemical groups most frequently found in contact allergens and the mechanism by which they react with nucleophilic groups. The previously defined three main types of mechanism, nucleophilic substitution on a saturated center (e.g., alkyl halides and epoxides), nucleophilic substitution on an unsaturated center (aromatic halides or esters), and nucleophilic addition (carbonyl derivatives and  $\alpha, \beta$ -unsaturated systems), can be seen.

### BACK TO CONTACT ALLERGY

If we consider the human body in its entirety and from a chemical viewpoint, it becomes apparent that a very large proportion of biological structures, especially nucleic acids and proteins, contain many electron-rich groups (those containing nitrogen, phosphorus, oxygen, or sulfur). We can thus consider the human body as being overall nucleophilic. It is therefore not surprising that many biological mechanisms are disturbed on contact with electrophilic chemical substances. Depending on the site of action of these electrophilic molecules, the effect can be mutagenic (Frierson et al., 1985), toxic (Guengerich and Liebler, 1985), or allergenic if the target is the skin. In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens (Figure 3). Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine, can react with electrophiles (Means and Feeney, 1971). Thus it has been shown by nuclear magnetic resonance (NMR) that nickel sulfate was interacting with histidine residues of peptides bound to a major histocompatibility complex (MHC) molecule (Romagnoli et al., 1991) and that methyl alkanesulfonates, allergenic methylating agents, were mainly reacting with histidine and to a less extent with lysine, methionine, cysteine, and tyrosine (Lepoittevin and Benezra, 1992). If we consider the chemical structure of some allergens (Figure 4) in the light of the chemical principles already outlined, it is easy to understand that all of these molecules will be able to react with biological nucleophiles, certain amino acids in proteins, to form extremely stable covalent bonds and thus lead to the triggering of delayed hypersensitivity. Again, the previously described three main types of mechanism for the formation of covalent bonds are seen; the arrows indicate the reactive center of each molecule.

In recent years, the radical mechanism has gained increased interest in the discussion of the mechanism of hapten-protein binding (Schmidt et al., 1990). This mechanism, which has never been firmly established, has been postulated to explain, for example, the allergenic potential of eugenol versus *iso*-eugenol (Barrat and Basketter, 1992). More recently, studies indicating that radical reactions were important for haptens containing allylic hydroperoxide groups have been published (Gäfvert et al., 1994; Lepoittevin and Karlberg, 1994).

### THE HAPTEN-PROTEIN BOND: COVALENT OR NONCOVALENT?

In biology, few phenomena are irreversible, with the majority being controlled by equilibria. It is easy to understand that the more stable the hapten-protein complex, the greater is the possibility of the immune system being able to process the immunological information, resulting in allergy. Given this, we can understand why the very strong and difficult-to-reverse covalent

Table 1. Principal electrophilic groups seen in contact allergy, with mechanisms of reaction with nucleophiles and the products

Group	Name	Reaction mechanism	Product
$R-CH_2-X$ $X = Cl, Br, I$	Alkyl halide	Nucleophilic substitution on a saturated center	$Nu-CH_2-R$
	Aryl halide	Nucleophilic substitution on an unsaturated center	
$X = F, Cl, Br, I$			
	Aldehyde; $R' = H$	Nucleophilic addition	
	Ketone; $R' = \text{alkyl or aryl}$		
	Ester; $R' = OR$	Nucleophilic substitution on an unsaturated center	
	Amide; $R' = NHR$		
	Epoxide	Nucleophilic substitution on a saturated center	
	Lactone; $X = O$	Nucleophilic substitution on an unsaturated center	
	Lactame; $X = NH$		
	Unsaturated aldehydes and ketones	Nucleophilic addition	
	<i>para</i> -Quinone	Nucleophilic addition	
	<i>ortho</i> -Quinone	Nucleophilic addition	
$Ni^{++}, Co^{++}, Cr^{IV}$	Metal salts	Coordination bonds	

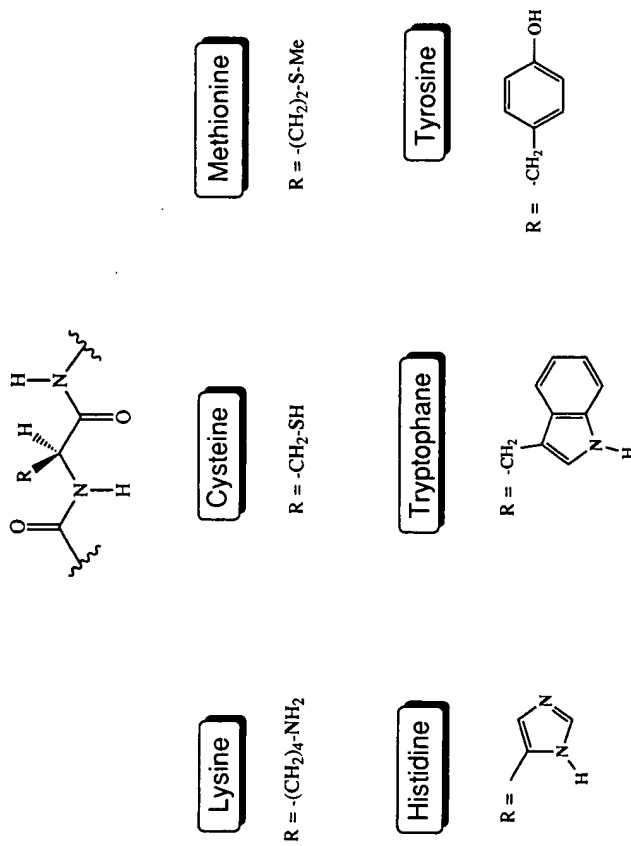


Figure 3. Principle nucleophilic residues in proteins.

bond produces a maximal biological efficacy. It is therefore natural that it is this type of bond that is found in the majority of cases of allergy. However, it would be incorrect to think that only covalent bonds result in allergy. In the case of metal salts, the formation of a covalent bond is impossible, and it is clear that a sufficiently stable coordination complex must be formed between the metal salt and the electron-rich residues of proteins (Polak and Frey, 1973; Hutchinson et al., 1975). These coordination complexes are therefore sufficiently stable, and the protein modification sufficiently important, to lead to allergy (Sinigaglia, 1994).

## METABOLISM AND PROHAPTENS

Far from being an inert tissue, the skin is the site of many metabolic processes, which can result in structural modification of xenobiotics that penetrate into it. These metabolic processes, primarily intended for the elimination of foreign molecules during detoxification, can, in certain cases, convert harmless molecules into derivatives with electrophilic, and therefore allergenic, properties. The metabolic processes are mainly based on oxidation reactions via extremely powerful enzymatic hydroxylation systems, such as the cytochrome P-450 enzymes (Mansuy, 1985), but monoamine oxidases, which convert amines to aldehydes, and peroxidases seem to play an important role in the metabolism of haptens. When activated by the production of hydrogen peroxide during the oxidative stress following the introduction of a xenobiotic into the skin, peroxidases convert the electron-rich aromatic derivatives (aminated or hydroxylated) into quinones, which are powerful electrophiles. In this way, the long-chain catechols, responsible for the severe allergies to poison ivy (*Rhus radicans* L.) and poison oak (*Rhus diversiloba* T.), are oxidized in vivo to the highly reactive orthoquinones (Dupuis, 1979) (Figure 5). The same applies to paraphenylenediamine or hydroquinone derivatives, such as the allergens from

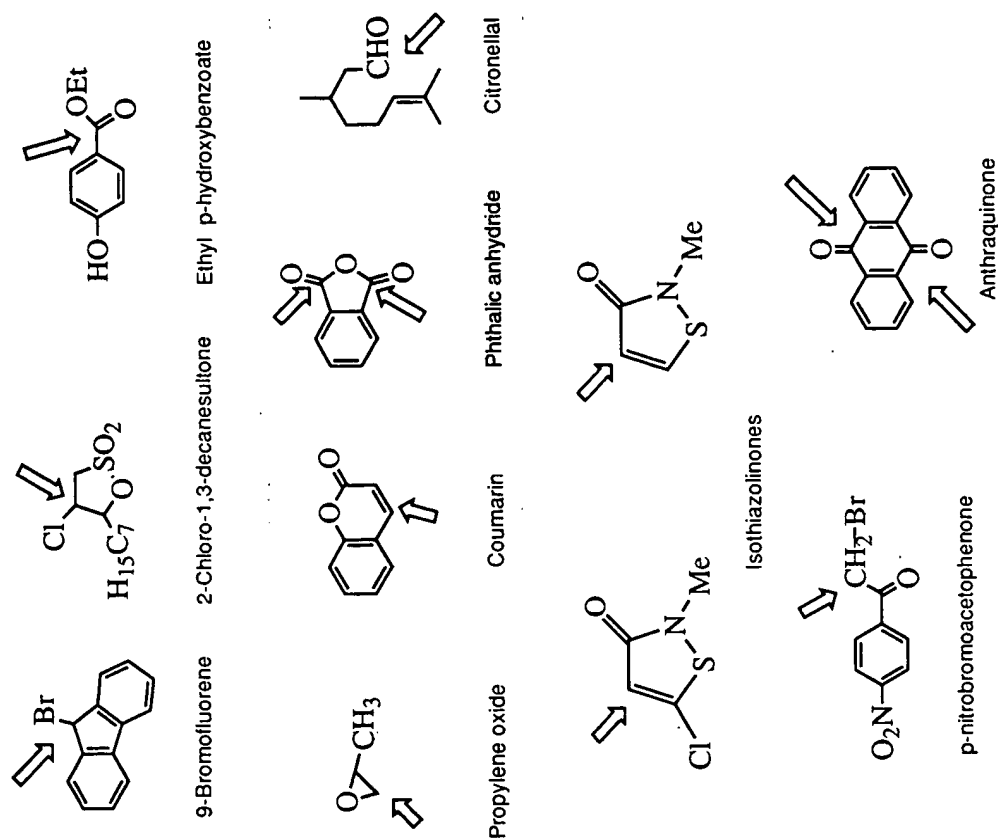


Figure 4. Examples of allergizing molecules. The electrophilic center is indicated by an arrow.

*Phacelia crenulata* Torr. (Reynolds and Rodriguez, 1981), which are converted into electrophilic paraquinones. Metabolic reactions involving enzymatic hydrolyses can also occur in the skin. It is thus that the tuliposides A and B, found in the bulb of the tulip (*Tulipa gesneriana* L.), are hydrolyzed, releasing the actual allergens, tulipalines A and B (Bergmann et al., 1967).

All these molecules, which have themselves no electrophilic properties and cannot therefore be haptens but which can be metabolized to haptens, are referred to as prohaptens (Landsteiner and Jacobs, 1936; Dupuis and Benezra, 1982) and play an important role in contact allergy because of their number and their highly reactive nature. The fact that the structure of the metabolized molecule can be far removed from the structure of the initial molecule can make allergologic investigations even more difficult.

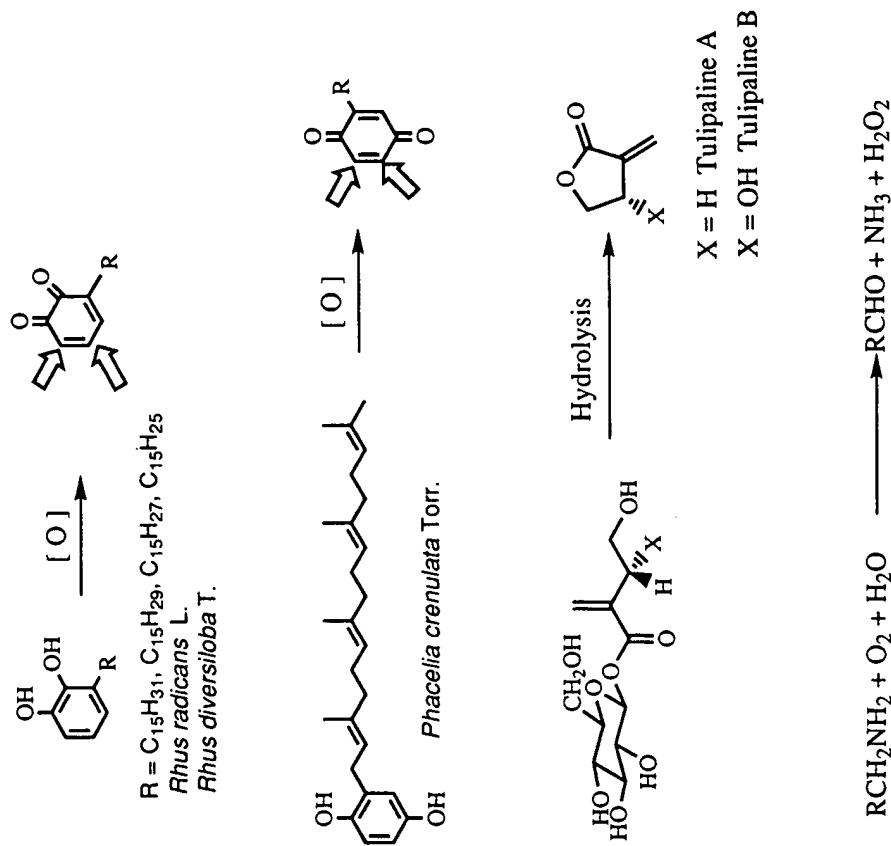


Figure 5. Examples of metabolism of molecules into contact allergens.

Nonenzymatic processes, such as reaction with atmospheric oxygen or ultraviolet irradiation, can also induce changes in the chemical structure of molecules. Many terpenes spontaneously auto-oxidize in air, producing allergizing derivatives. In the 1950s it was found that allergenic activity of turpentine was mainly due to hydroperoxides of one of the monoterpene  $\Delta^3$ -carene (Hellerström et al., 1955). This is also the case for abietic acid, the main constituent of colophony, which is converted into the highly reactive substance hydroperoxide (Karlborg, 1988) by contact with air (Figure 6). Such an auto-oxidation mechanism has also been demonstrated for another monoterpene, *d*-limonene, found in citrus fruits. *d*-Limonene itself is not allergenic, but at air exposure hydroperoxides, epoxides, and ketones are formed that are strong allergens (Karlborg et al., 1994).

#### HAPTENS AND CROSS-ALLERGY

The factors that control molecular recognition during the elicitation stage are primarily the nature of the chemical group and the compatibility of the spatial geometry. Although the first

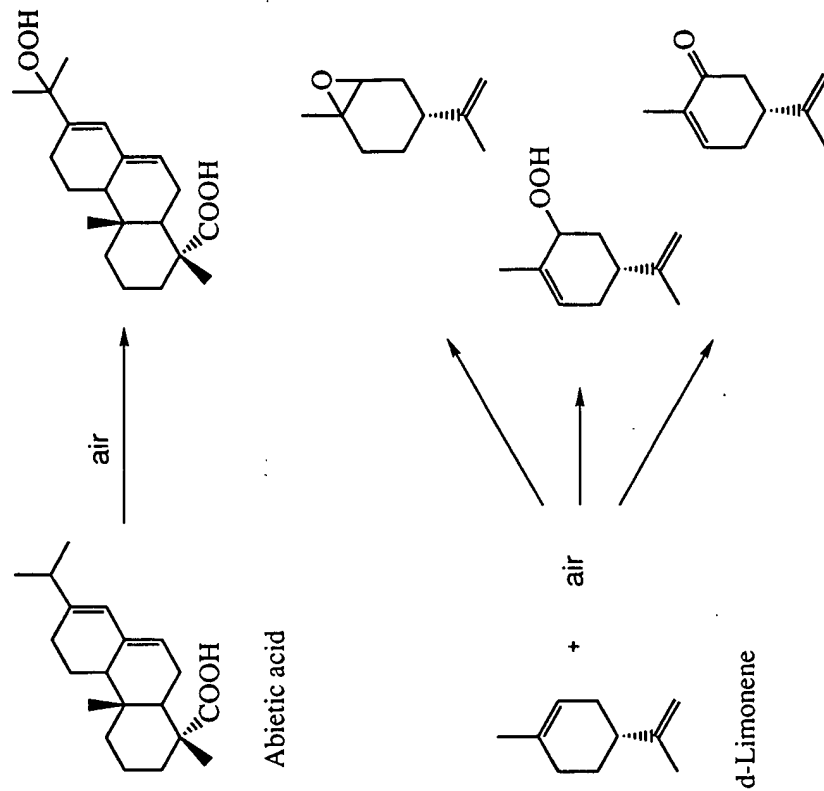


Figure 6. Examples of chemical modification by reaction with air.

factor (the identity of the chemical group) is very important and serves to define what are commonly called the group allergies, it cannot account for all structure-activity relationships. Receptor molecules are highly sensitive to volume and shape, and molecules must have a similar size and spatial geometry to be recognized by the same receptor. Thus, even though the molecules tulipaline A or B and alantolactone (the allergen of *Inula helenium* L.) bear the same chemical group,  $\alpha$ -methylene- $\gamma$ -butyrolactone, they cannot give rise to cross-allergic reactions, as their spatial volumes are too different (Figure 7). In contrast, isoalantolactone and alantolactone produce a cross-allergic reaction (Stampf et al. 1982), since they share a homologous chemical group and spatial volume. The term cross-allergy is often misused and should be restricted to the well-defined cases that can be called the true cross-allergies (Baer, 1954; Benezra and Maibach, 1984).

True cross-allergy between a sensitizer A and a triggering agent B can be interpreted in various ways:

- A and B are chemically and structurally similar.
- A is metabolized to a compound that is similar to B.
- B is metabolized to a compound that is similar to A.
- A and B are metabolized to similar compounds.

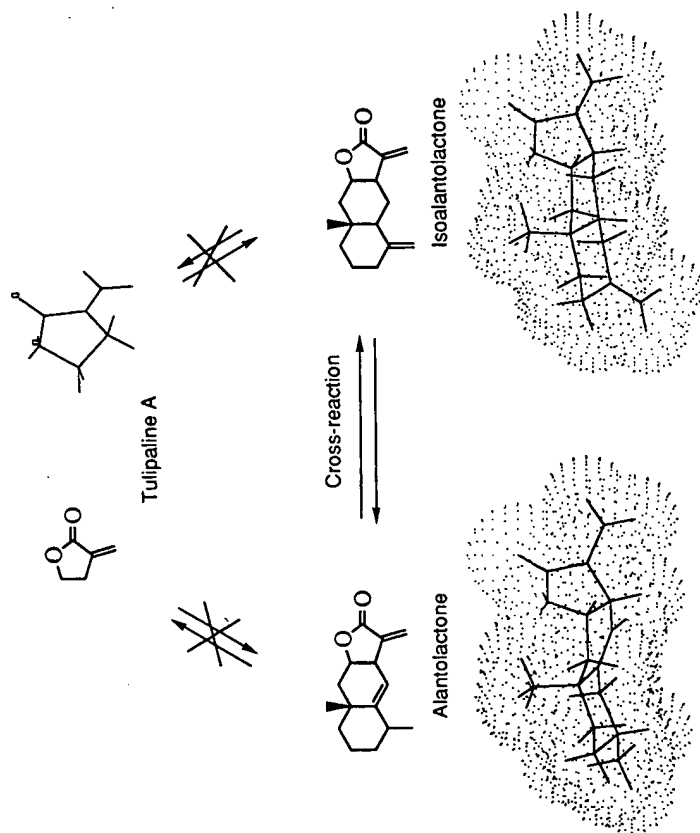


Figure 7. Chemical structure and spatial representation of tulipaline A, alantolactone, and isosalantolactone.

The identification of cross-allergenic responses can be especially difficult, particularly in humans, in whom the possibility of co- or polysensitization should never be ruled out. In addition, the metabolism of molecules can be very complex, and two molecules with a priori little in common can be converted to derivatives that have a similar structure. Thus, derivatives of hydroquinones and *para*-phenylenediamines can be converted into benzoquinone derivatives. It is therefore dangerous to draw conclusions from tests without knowing how the substances used are liable to be metabolized. Many reactions described as demonstrating cross-allergy are, without doubt, due to co-sensitization (Benezra and Maibach, 1984). Experimental studies in animals are often the only means of being really certain of what happens during recognition. The concept of the prohaptens is very important in the interpretation of results in allergy. As the structure of the metabolized molecule can sometimes be very different from that of the initial molecule, it can be difficult to establish similarities of chemical groups and structure.

#### Molecular Modeling as a Tool for Cross-Reactivity Analysis

In the last few years, molecular modeling has been shown to be a powerful tool in studies of conformation-dependent drug-receptor interactions and structure-activity relationship analysis (Cohen et al., 1990). Despite the great potential of this technique, few attempts to analyze cross-reaction patterns in the field of allergic contact dermatitis have yet been reported. One reason may be the heterogeneous population of patients with heterogeneous clinical histories, in which it is somewhat difficult to distinguish between actual cross-reaction and concomitant sensitization. A second reason is that, to be effective, structure-activity relationship studies

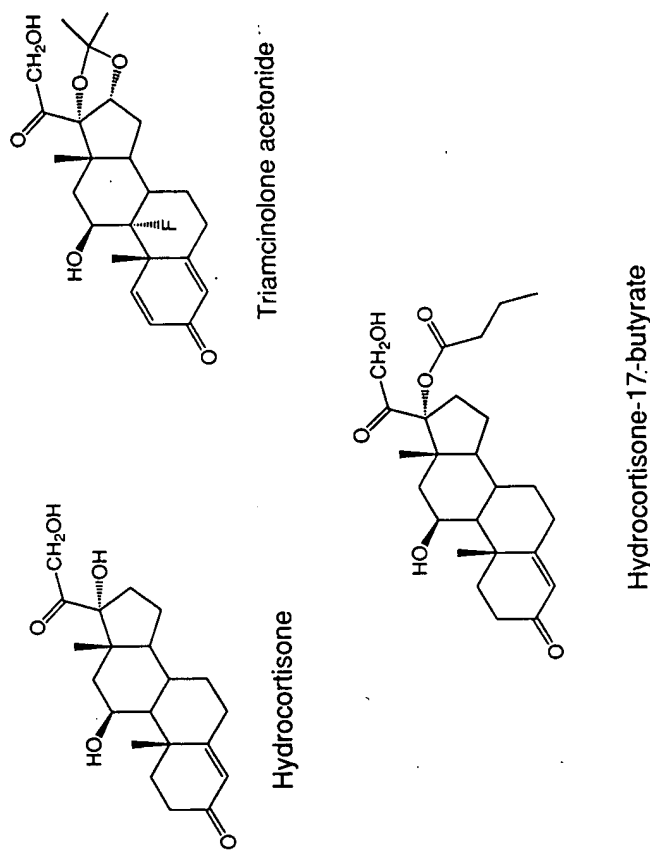


Figure 8. Chemical structure of hydrocortisone (class A), triamcinolone acetonide (class B), and hydrocortisone 17-butyrate (class D).

need data for a wide range of molecules. The clinical investigation of contact dermatitis to corticosteroids, in which a large number of related substances are tested on a large number of patients, represents a good opportunity to carry out such a structure-activity study. From the statistical analysis of the clinical data, it is now possible to advance an experimentally supported hypothesis for cross-reaction patterns. Coopman et al. (1989) hypothesized that cross-reactions occur primarily within certain groups of corticosteroids. They distinguished four groups, group A consisting of hydrocortisone, tixocortol pivalate, and related compounds, group B consisting of triamcinolone acetonide, amcinonide, and related compounds, group C consisting of betamethasone, dexamethasone, and related compounds, and group D consisting of esters such as hydrocortisone-17-butyrate and clobetasone-17-butyrate (Figure 8). It is now possible to correlate this with conformational characteristics and to establish a molecular basis for cross-reaction patterns in patient sensitized to corticosteroids. This could be invaluable in the prediction of potential cross-reactions to new molecules.

#### Example of Conformational Analysis: Cross-Reaction to Corticosteroids

The conformation of corticosteroids from groups A, B, C, and D was analyzed (Lepoittevin et al., 1994). This study was based on two hypotheses. The first was that all corticosteroids should interact with proteins in a very similar way. All corticosteroid molecules were designed to interact with the same type of receptors, and thus should be more or less metabolized in similar ways. The second hypothesis, based on chemical observations, was that esters at position 21 are readily hydrolyzed to give the free alcohol while esters at position 17 are more resistant to hydrolysis, due to a strong steric hindrance. Thus, for example, tixocortol pivalate was

considered as tixocortol with a free thiol group at position 21, and alclometasone 17,21-dipropionate was considered as alclometasone 17-propionate.

All molecules were drawn from energy-minimized building blocks and were then submitted to a multiconformational analysis in order to achieve the most energetically stable conformation. These conformations were then compared for analogies or differences in the van der Waals volumes that define the electronic shape of the molecule. As expected from the hypothesis, significant group-specific characteristics of volume and shape were found for molecules of group A, B, and D but not for molecules of group C.

In terms of molecular characteristics, the existence of groups A, B, and D, as defined by the analysis of cross-reaction patterns in patients sensitized to corticosteroids, is fully supported by the conformational analysis of these molecules. Molecules of the same group have very similar spatial structures, explaining the cross-reactions observed. In addition, molecules from one group are sufficiently different from molecules of another group to explain the lack of cross-reactions observed between groups A, B, and D.

The volume occupied by specific groups on the  $\alpha$  face of ring D seems to be critical for the molecular recognition of corticosteroids by receptors of immunocompetent cells, while modifications of other parts of the molecule seem to have little effects on the recognition patterns. As shown in Figure 9, each group represents a well-defined, characteristic shape that can be very useful for the prediction of potential cross-reactions of new corticosteroid molecules.

## CONCLUSION

The principles that we have just discussed permit a rational approach to the phenomena of contact allergy, but, in actual fact, we often have available only indirect evidence suggestive of one mechanism or another. Although the chemical bases for hapten-protein interactions can be checked in the laboratory by the use of nucleophilic amino acids, small peptides, and model proteins, and although a certain number of steps can be checked, at the present time, no method is available to follow a hapten step by step during the entire immunological process leading to contact allergy. Many points await investigation, but in many cases a "chemical" analysis of the problem does allow us to understand and to foresee cross-allergies and thus to warn the patient about structurally related products.

## REFERENCES

- Baer, R. L. 1954. Cross-sensitization phenomena. In *Modern trends in dermatology*, ed. R. M. B. Mackenna, pp. 232-238. London: Butterworth.
- Barratt, M. D., and Basketter, D. A. 1992. Possible origin of the skin sensitization potential of eugenol and related compounds. *Contact Dermatitis* 27:98-104.
- Benezra, C., and Maibach, H. 1984. True cross-sensitization, false cross-sensitization and otherwise. *Contact Dermatitis* 11:65-69.
- Bergmann, H. H., Beijersbergen, J. C. H., Overeem, J. C., and Stijpesteijn, A. K. 1967. Isolation and identification of  $\alpha$ -methylene- $\gamma$ -butyrolactone: A fungitoxic substance from tulips. *Rec. Trav. Chim. Pays-Bas* 86:709-713.
- Cohen, N. C., Blaney, J. M., Humblet, C., Gund, P., and Barry, D. C. 1990. Molecular modeling software and methods for medicinal chemistry. *J. Med. Chem.* 33:883-984.
- Coopman, S., Degreef, H., and Dooms-Goossens, A. 1989. Identification of cross-reaction patterns in allergic contact dermatitis from topical corticosteroids. *Br. J. Dermatol.* 121:27-34.
- Darley, M. O., Post, W., and Munter, R. L. 1977. Induction of cell-mediated immunity to chemically modified antigens in guinea pigs. *J. Immunol.* 118:963-970.
- Dupuis, G. 1979. Studies of poison ivy. In vitro lymphocytes transformation by urushiol protein conjugates. *Br. J. Dermatol.* 101:617-624.
- Dupuis, G., and Benezra, C. 1982. *Allergic contact dermatitis to simple chemicals*. New York: Marcel Dekker.
- Frierson, M. R., Klopman, G., and Rosenkranz, H. S. 1985. Structure activity relationship among mutagens and carcinogens: A review. *Environ. Mutagen.* 8:283.

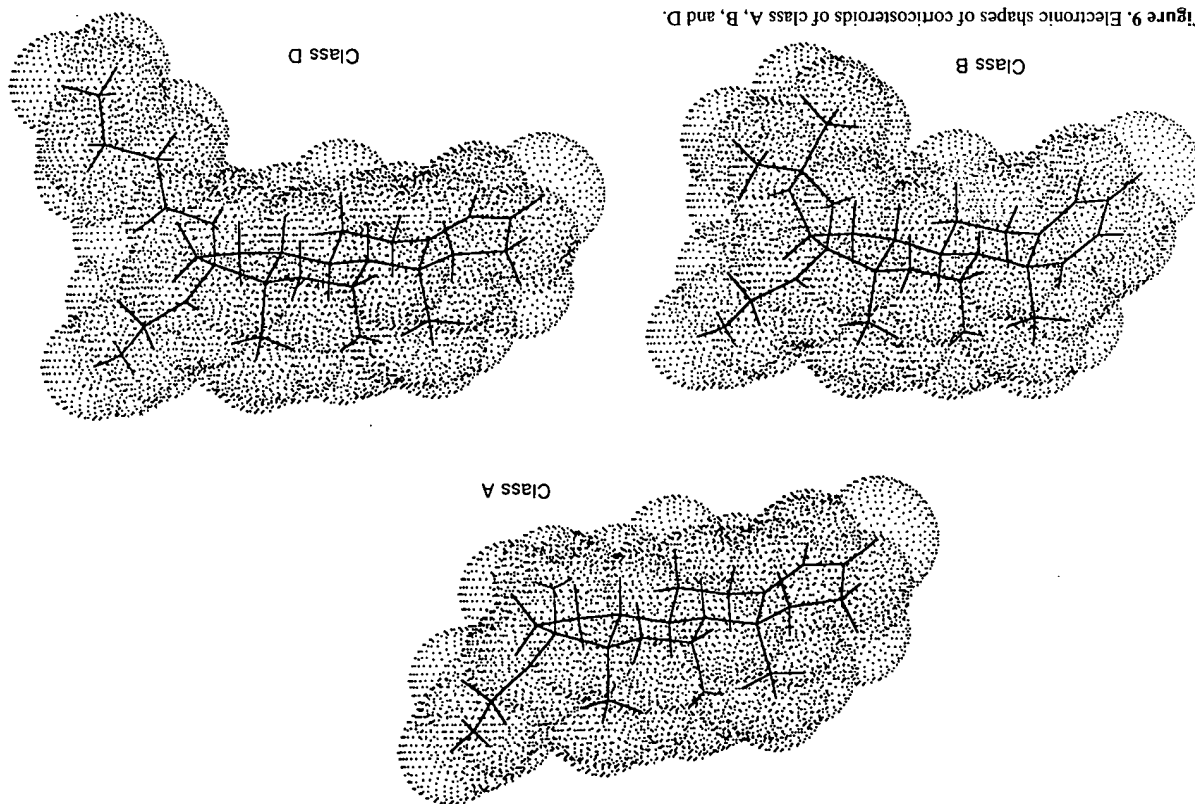


Figure 9. Electronic shapes of corticosteroids of class A, B, and D.

- Gälfvert, E., Shao, L. P., Karlberg, A.-T., Nilsson, U., and Nilsson, J. L. G. 1994. Contact allergy to resin acid hydroperoxides. Hapten binding via free radicals and epoxides. *Chem. Res. Toxicol.* 7:260-266.
- Guengerich, F. P., and Liebler, D. C. 1985. Enzymatic activation of chemicals. *CRC Crit. Rev. Toxicol.* 14:259-307.
- Hellerström, S., Thyresson, N., Blohm, S.-G., and Widmark, G. 1955. On the nature of eczematogenic component of oxidized  $\Delta^3$ -carene. *J. Invest. Dermatol.* 24:217-224.
- Hotchkiss, S. A. M. 1992. Skin as a xenobiotic metabolizing organ. In *Progress in drug metabolism*, ed. G. G. Gibson, Vol. 13, pp. 217-262. London: Taylor and Francis.
- Hutchinson, F., McLeod, T. M., and Raffle, E. J. 1975. Nickel hypersensitivity. Nickel binding to aminoacids and lymphocytes. *Br. J. Dermatol.* 93:557-561.
- Karlberg, A.-T. 1988. Contact allergy to colophony. Chemical identification of allergens. Sensitization experiments and clinical experiments. *Acta Dermatovenerol.* 68(Suppl. 139):1-43.
- Karlberg, A.-T., Shao, L. P., Nilsson, U., Gälfvert, E., and Nilsson, J. L. G. 1994. Hydroperoxides in oxidized d-limonene identified as potent contact allergens. *Arch. Dermatol. Res.* 286:97-103.
- Landsteiner, K., and Jacobs, J. L. 1936. Studies on the sensitization of animals with simple chemicals. *J. Exp. Med.* 64:625-639.
- Lepoittevin, J.-P., and Benezra, C. 1992.  $^{13}\text{C}$  enriched methyl alkanesulfonates: New lipophilic methylating agents for the identification of nucleophilic amino acids of proteins by NMR. *Tetrahedron Lett.* 33:3875-3878.
- Lepoittevin, J.-P., and Karlberg, A.-T. 1994. Interaction of allergenic hydroperoxides with proteins: A radical mechanism. *Chem. Res. Toxicol.* 7:130-133.
- Lepoittevin, J.-P., Drieghe, J., and Dooms-Goossens, A. 1995. Studies in patients with corticosteroid contact allergy: Understanding cross-reactivity among different steroids. *Arch. Dermatol.* 131:31-37.
- Mansuy, D. 1985. Particular ability of cytochrome P450 to form reactive intermediates and metabolites. In *Drug metabolism: Molecular approaches and pharmacological implications*, ed. G. Sisti, pp. 195-203. New York: Pergamon.
- Means, G. E., and Feeney, R. E. 1971. *Chemical modification of proteins*. San Francisco: Holden Day.
- Polak, L., and Frey, J. R. 1973. Studies on contact hypersensitivity to chromium in the guinea pig. *Int. Arch. Allergy Appl. Immunol.* 44:51-61.
- Potts, R. O., and Guy, R. 1992. Predicting skin permeability. *Pharmacol. Res.* 9:663-669.
- Reynolds, G., and Rodriguez, E. 1981. Prenylated hydroquinones: Contact allergens from trichomes of *Phacelia minor* and *P. parryi*. *Phytochemistry* 20:1365-1366.
- Romagnoli, P., Labhardt, A. M., and Sinigaglia, F. 1991. Selective interaction of nickel with an MHC bound peptide. *EMBO J.* 10:1303-1306.
- Schmidt, R., Kahn, L., and Chung, L. Y. 1990. Are free radicals and quinones the haptenic species derived from urushiols and other contact allergenic mono- and dihydric alkylbenzenes? The significance of NADH, glutathione and redox cycling in the skin. *Arch. Dermatol. Res.* 282:56-64.
- Sinigaglia, F. 1994. The molecular basis of metal recognition by T cells. *J. Invest. Dermatol.* 102:398-401.
- Stampf, J.-L., Benezra, C., Klecak, G., Geleick, H., Schulz, K. H., and Hausen, B. 1982. The sensitization capacity of helenin and two of its main constituents, the sesquiterpene lactones, alantolactones and isolantalactone. *Contact Dermatitis* 8:16-24.

## Chapter Thirteen

### SYSTEMIC CONTACT-TYPE DERMATITIS

Torkil Menné, Niels Veien, and Howard I. Maibach

#### INTRODUCTION

Systemic contact dermatitis is an inflammatory skin disease that may occur in contact-sensitized individuals when these persons are exposed to the hapten orally, transcutaneously, intravenously, or by inhalation. The entity can be present with clinically characteristic features or be clinically indistinguishable from other types of contact dermatitis. Contact sensitization to ubiquitous haptens is common. In a recent Danish population-based study, 15.2% reacted to one or more of the haptens in the Standard patch test series (Nielsen and Menné, 1992). The total number of individuals at risk of developing a systemic contact dermatitis reaction is therefore large. While systemic contact dermatitis reaction from medicaments is a well-established entity, a systemic reaction from metals, particularly nickel, is still regarded as controversial (Burrows, 1992; Möller, 1993).

The first description of systemic contact dermatitis can probably be ascribed to the pioneering British dermatologist Thomas Bateman (Shelley and Crissey, 1970). His description of the mercury eczema called eczema rubrum is similar to what we today describe as the baboon syndrome:

Eczema rubrum is preceded by a sense of stiffness, burning, heat and itching in the part where it commences, most frequently the upper and inner surface of the thighs and about the scrotum in men, but sometimes it appears first in the groins, axillae or in the bends of the arms, on the wrists and hands or on the neck.

In this century the systemic spread of nickel dermatitis was described by Schittenhelm and Stockinger in Kiel in 1925. By patch testing nickel-sensitive workers with nickel sulfate, they observed the spread of dermatitis and flares in the original areas of contact dermatitis. The literature on systemic contact dermatitis is now comprehensive. Recent reviews include Cronin (1980), Fisher (1986), and Veien et al. (1990).

#### CLINICAL FEATURES

The clinical symptoms related to systemic contact dermatitis are summarized in Table 1. The symptoms may appear exclusively on the skin, but general symptoms are occasionally seen. Knowledge of the clinical symptoms stems from clinical observations and experimental oral challenge studies.

Flare-up reactions in the primary site of dermatitis or previously positive patch-test sites raise the suspicion of systemic contact dermatitis (Ekelund and Möller, 1969; Christensen and Möller, 1975; Menné and Weismann, 1984). Flare-up of previously positive patch-test sites following ingestion of the hapten is a fascinating and specific sign of a systemic contact





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Dynamic diversity and **small-molecule** evolution: a new paradigm for ligand identification.

Trends Biotechnol. 1999 May;17(5):205-9. Review.

PMID: 10322446 [PubMed - indexed for MEDLINE]

☐ **2642:** [Smith RG, Feighner S, Prendergast K, Guan X, Howard A.](#) Related Articles, Lin

A New Orphan Receptor Involved in Pulsatile Growth Hormone Release

Trends Endocrinol Metab. 1999 May;10(4):128-135.

PMID: 10322406 [PubMed - as supplied by publisher]

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Anti-sigma factors.

Curr Opin Microbiol. 1999 Apr;2(2):135-41. Review.

PMID: 10322161 [PubMed - indexed for MEDLINE]

☐ **2644:** [Boehm MF, Fitzgerald P, Zou A, Elgort MG, Bischoff ED, Mere L, Mais DE, Bissonnette RP, Heyman RA, Nadzan AM, Reichman M, Allegretto EA.](#) Related Articles, Lin

Novel nonsecosteroidal vitamin D mimics exert VDR-modulating activities with less calcium mobilization than 1,25-dihydroxyvitamin D3

Chem Biol. 1999 May;6(5):265-75.

PMID: 10322128 [PubMed - indexed for MEDLINE]

☐ **2645:** [Kwong AD, Kim JL, Rao G, Lipovsek D, Raybuck SA.](#) Related Articles, Lin

Hepatitis C virus NS3/4A protease.

Antiviral Res. 1999 Feb;41(1):67-84. Review.

PMID: 10321580 [PubMed - indexed for MEDLINE]

☐ **2646:** [Zhang B, Salituro G, Szalkowski D, Li Z, Zhang Y, Royo I, Vilella D, Diez MT, Pelaez F, Ruby C, Kendall RL, Mao X, Griffin P, Calaycay J, Zierath JR, Heck JV, Smith RG, Moller DE.](#) Related Articles, Lin

Discovery of a **small molecule** insulin mimetic with antidiabetic activity in mice.

Science. 1999 May 7;284(5416):974-7.

PMID: 10320380 [PubMed - indexed for MEDLINE]

☐ **2647:** [Baba M, Nishimura O, Kanzaki N, Okamoto M, Sawada H, Iizawa Y, Shiraishi M, Aramaki Y, Okonogi K, Ogawa Y, Meguro K, Fujino M.](#) Related Articles, Lin

A **small-molecule** nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity.

Proc Natl Acad Sci U S A. 1999 May 11;96(10):5698-703.

PMID: 10318947 [PubMed - indexed for MEDLINE]

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Opportunities for novel therapeutic agents acting at chemokine receptors  
Drug Discov Today. 1999 Feb;4(2):80-92.  
PMID: 10234160 [PubMed - as supplied by publisher]

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Characterization of the GlnK protein of Escherichia coli.  
Mol Microbiol. 1999 Apr;32(2):301-13.  
PMID: 10231487 [PubMed - indexed for MEDLINE]

- ☐ **2650:** [Groarke JM, Pevear DC.](#) Related Articles, Lin



Attenuated virulence of pleconaril-resistant coxsackievirus B3 variants.  
J Infect Dis. 1999 Jun;179(6):1538-41.  
PMID: 10228078 [PubMed - indexed for MEDLINE]

- ☐ **2651:** [Ficheux A, Argiles A, Bosc JY, Mion C.](#) Related Articles, Lin



Analysis of the influence of the infusion site on dialyser clearances measured in an in vitro system mimicking haemodialysis and haemodiafiltration.  
Blood Purif. 1999;17(1):10-8.  
PMID: 10224334 [PubMed - indexed for MEDLINE]

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Multiple activation states of integrin alpha4beta1 detected through their different affinities for a small molecule ligand.  
J Biol Chem. 1999 May 7;274(19):13167-75.  
PMID: 10224072 [PubMed - indexed for MEDLINE]

- ☐ **2653:** [Burkhard P, Hommel U, Sanner M, Walkinshaw MD.](#) Related Articles, Lin



The discovery of steroids and other novel FKBP inhibitors using a molecular docking program.  
J Mol Biol. 1999 Apr 16;287(5):853-8.  
PMID: 10222195 [PubMed - indexed for MEDLINE]

- ☐ **2654:** [Aggen JB, Humphrey JM, Gauss CM, Huang HB, Nairn AC, Chamberlin AR.](#) Related Articles, Lin



The design, synthesis, and biological evaluation of analogues of the serine-threonine protein phosphatase 1 and 2A selective inhibitor microcystin LA: rational modifications imparting PP1 selectivity.  
Bioorg Med Chem. 1999 Mar;7(3):543-64.  
PMID: 10220039 [PubMed - indexed for MEDLINE]

- ☐ **2655:** [Baylink DJ, Strong DD, Mohan S.](#) Related Articles, Lin



The diagnosis and treatment of osteoporosis: future prospects.  
Mol Med Today. 1999 Mar;5(3):133-40. Review.  
PMID: 10203737 [PubMed - indexed for MEDLINE]

- ☐ **2656:** [McCarthy JR, Heinrichs SC, Grigoriadis DE.](#) Related Articles, Lin



Recent advances with the CRF1 receptor: design of small molecule inhibitors, receptor subtypes and clinical indications.  
Curr Pharm Des. 1999 May;5(5):289-315. Review.  
PMID: 10213797 [PubMed - indexed for MEDLINE]

- ☐ **2657:** [Felix JP, Bugianesi RM, Schmalhofer WA, Borris R, Goetz MA, Hensens OD, Bao JM, Kayser F, Parsons WH, Rupprecht K, Garcia ML, Kaczorowski GJ, Slaughter RS.](#) Related Articles, Link



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Biochemistry. 1999 Apr 20;38(16):4922-30.

PMID: 10213593 [PubMed - indexed for MEDLINE]

- ☐ **2658:** [Haque TS, Skillman AG, Lee CE, Habashita H, Gluzman IY, Ewing TJ, Goldberg DE, Kuntz ID, Ellman JA.](#) Related Articles, Link



Potent, low-molecular-weight non-peptide inhibitors of malarial aspartyl protease plasmepsin II.

J Med Chem. 1999 Apr 22;42(8):1428-40.

PMID: 10212129 [PubMed - indexed for MEDLINE]

- ☐ **2659:** [Hu LY, Ryder TR, Rafferty MF, Cody WL, Lotarski SM, Miljanich GP, Millerman E, Rock DM, Song Y, Stoehr SJ, Taylor CP, Weber ML, Szoke BG, Vartanian MG.](#) Related Articles, Link



N,N-dialkyl-dipeptidylamines as novel N-type calcium channel blockers

Bioorg Med Chem Lett. 1999 Mar 22;9(6):907-12.

PMID: 10206559 [PubMed - indexed for MEDLINE]

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Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein.

Proc Natl Acad Sci U S A. 1999 Apr 13;96(8):4438-42.

PMID: 10200280 [PubMed - indexed for MEDLINE]

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## Analysis of the influence of the infusion site on dialyser clearance measured in an in vitro system mimicking haemodialysis and haemodiafiltration.

Ficheux A, Argiles A, Bosc JY, Mion C.

UDSA-AIDER, Montpellier, France.

**BACKGROUND:** Blood flow (QB), dialysate flow (QD), and dialyser characteristics are the three major factors driving dialysis efficacy. Haemodiafiltration has added an increased convective volume to increase efficacy. We aimed to assess the influence of the infusion site of the replacement fluid in an in vitro system emulating haemodiafiltration. **METHODS:** An in vitro system allowing us to control the dialysate temperature, concentration gradient, the flow of both dialyser sides over a range wider than that compatible with clinic, was set to evaluate the influence of the different parameters on dialysis efficacy. The total ion clearance was used as an accepted method for small molecule clearance assessment. Cellulose triacetate (CT190C, Baxter; FB170U, Nipro) and polysulfone (HF80, Fresenius) dialysers were included in the study. Dialysis as well as on-line diafiltration both with pre- and postdilutional infusion were assessed. The experimental conditions presented in this study included QD 620 and 970 ml/min. The convective flows ranged from 50 to 200 ml/min. **RESULTS:** For QD = 620 ml/min and a QB = 350 ml/min the total ion clearance ranged from 269 to 274 for HF80, from 291 to 294 for FB170 and from 294 to 302 for CT190. The variability of the measurements was very low (SD < 1%). Total ion clearance increased by 17-21% when QB was raised from 300 to 400 ml/min. Increasing QD from 420 to 970 ml/min (for QB = 350 ml/min), resulted in an increase in total ion clearance which was more marked at lower QD (from 420 to 620 ml/min) and plateaued thereafter (from 620 to 970 ml/min). Postdilutional on-line diafiltration with 100 ml/min of infusate resulted in an additional increase in total ion clearance of 5.4-8.6%. This increase was proportional to the infused volume. On the contrary, predilution on-line diafiltration resulted in a decrease in total ion clearance which was also proportional to the infused volume (between -5.1 and -6.9% at 100 ml/min infusion volume and -9.7 to -12.9% at 200 ml/min). **CONCLUSIONS:** The present in vitro system provided accurate and reproducible results on dialyser clearances. Our experiments confirmed previous studies on the influence of (

and QD on dialyser efficacy. Further, they show that the proportional increase in postdilutional on-line diafiltration is lesser than that previously reported. More importantly, they also show that pre-dilution infusion in high efficiency systems results in a drop in dialyser clearance compared to dialysis alone, as proportional to the infusion rate. Thus, increasing the convective flow may increase dialysis efficacy even more than increasing QD alone. However, the choice of infusion site is crucial to obtaining this benefit in small molecule clearances.

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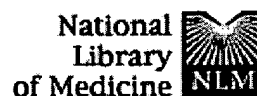
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[www.jbc.org](http://www.jbc.org)**Multiple activation states of integrin alpha4beta1 detected through their different affinities for a small molecule ligand.****Chen LL, Whitty A, Lobb RR, Adams SP, Pepinsky RB.**

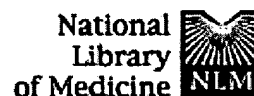
Biogen, Inc., Cambridge, Massachusetts 02142, USA.

We have used the highly specific alpha4beta1 inhibitor 4-((N'-2-methylphenylureido)-phenylacetyl-leucine-aspartic acid-valine-proline (BIO1211) as a model LDV-containing ligand to study alpha4beta1 integrin-ligand interaction on Jurkat cells under diverse conditions that affect the activation state of alpha4beta1. Observed KD values for BIO1211 binding ranged from a value 20-40 nM in the non-activated state of the integrin that exists in 1 mM Mg<sup>2+</sup> mM Ca<sup>2+</sup> to 100 pM in the activated state seen in 2 mM Mn<sup>2+</sup> to 18 pM when binding was measured after co-activation by 2 mM Mn<sup>2+</sup> plus 10 microgram/ml of the integrin-activating monoclonal antibody TS2/16. The large range in KD values was governed almost exclusively by differences in the dissociation rates of the integrin-BIO1211 complex, which ranged from 0.17 x 10<sup>(-4)</sup> s<sup>-1</sup> to >140 x 10<sup>(-4)</sup> s<sup>-1</sup>. Association rate constants varied only slightly under the same conditions, all falling in the narrow range from 0.9 to 2.7 x 10<sup>(6)</sup> M<sup>-1</sup> s<sup>-1</sup>. The further increase in affinity observed upon co-activation by divalent cations and TS2/16 compared with that observed at saturating concentrations of metal ions or TS2/16 alone indicates that the mechanism by which these factors bring about activation are distinct and identified a previously unrecognized high affinity state on alpha4beta1 that has not been detected by conventional assay methods. Similar changes in affinity were observed when the binding properties of vascular cell adhesion molecule 1 and CS1 to alpha4beta1 were studied, indicating that the different affinity states detected with BIO1211 are an inherent property of the integrin.

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**ELSEVIER SCIENCE  
FULL-TEXT ARTICLE****The discovery of steroids and other novel FKBP inhibitors using molecular docking program.****Burkhard P, Hommel U, Sanner M, Walkinshaw MD.**

Structural Biochemistry Unit, Edinburgh University, Michael Swann Building  
Edinburgh, Kings Buildings, EH9 3JR, UK.

The molecular docking computer program SANDOCK was used to screen small molecule three-dimensional databases in the hunt for novel FKBP inhibitors. Spectroscopic measurements confirmed binding of over 20 compounds to the target protein, some with dissociation constants in the low micromolar range. The discovery that FK506 binding protein is a steroid binding protein may be of wider biological significance. Two-dimensional NMR was used to determine the steroid binding mode and confirmed the interactions predicted by the docking program. Copyright 1999 Academic Press.

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FULL-TEXT ARTICLE**

**The design, synthesis, and biological evaluation of analogues of the serine-threonine protein phosphatase 1 and 2A selective inhibitor microcystin LA: rational modifications imparting PP1 selectivity.**

**Aggen JB, Humphrey JM, Gauss CM, Huang HB, Nairn AC, Chamberlin AR.**

Department of Chemistry, University of California at Irvine, 92697, USA.

Based on the results from previously reported molecular modeling analyses of the interactions between the inhibitor microcystin and the serine-threonine protein phosphatases 1 and 2A, we have designed analogues of microcystin I with structural modifications intended to impart PP1 selectivity. The synthesis of several first generation analogues followed by inhibition assays revealed that all three are PP1-selective, as predicted. Although the observed selectivities are modest, one of the designed analogues is more selective for PP1 than any known small molecule inhibitor.

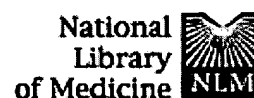
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## The diagnosis and treatment of osteoporosis: future prospects.

**Baylink DJ, Strong DD, Mohan S.**

Dept of Medicine, Loma Linda University, Loma Linda, CA 92354, USA.

baylinkd@llvamc.va.gov

Osteoporosis is a common disease that affects millions of patients throughout the world. We anticipate that both the diagnosis and the treatment of this disease will be revolutionized by the integration of genomics and informatics. It is predicted that a genetic algorithm will be developed to identify at-risk patients before they develop osteoporosis, so that preventive measures can be instituted. The sequencing of the human genome will lead to revolutionary advances in at least three areas of osteoporosis therapy: **small molecule** therapy, protein therapy and gene therapy. One area of focus for future therapeutics in osteoporosis will be on osteogenic agents, which should have high likelihood of success because the skeleton has the innate capacity to regenerate itself.

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**Recent advances with the CRF1 receptor: design of small molecule inhibitors, receptor subtypes and clinical indications.****McCarthy JR, Heinrichs SC, Grigoriadis DE.**

Neurocrine Biosciences, Inc., San Diego, CA 92121, USA.

jmccarthy@neuroscience.com

Corticotropin-releasing factor (CRF) has been widely implicated as playing a major role in modulating the endocrine, autonomic, behavioral and immune responses to stress. The recent cloning of multiple receptors for CRF as well as the discovery of non-peptide receptor antagonists for CRF receptors have begun a new era of CRF study. Presently, there are five distinct targets for CRF with unique cDNA sequences, pharmacology and localization. These fall into three distinct classes, encoded by three different genes and have been termed the CRF1 and CRF2 receptors (belonging to the superfamily of G-protein coupled receptors) and the CRF-binding protein. The CRF2 receptor exists as three splice variants of the same gene and have been designated CRF2a, CRF2b and CRF2g. The pharmacology and localization of all of these proteins in brain has been well established. The CRF1 receptor subtype is localized primarily in cortical and cerebellar regions while the CRF2a receptor is localized to subcortical regions including the lateral septum, and paraventricular and ventromedial nuclei of the hypothalamus. The CRF2b receptor is primarily localized to heart, skeletal muscle and in the brain, to cerebral arterioles and choroid plexus. The CRF2g receptor has most recently been identified in human amygdala. Expression of these receptors in mammalian cell lines has made possible the identification of non-peptide, high affinity, selective receptor antagonists. While the natural mammalian ligands oCRF and r/hCRF have high affinity for the CRF1 receptor subtype, they have lower affinity for the CRF2 receptor family making them ineffective labels for CRF2 receptors. [125I] Sauvagine has been characterized as a high affinity ligand for both the CRF1 and the CRF2 receptor subtypes and has been used in both radioligand binding and receptor autoradiographic studies as a tool to aid in the discovery of selective small molecule receptor antagonists. A number of non-peptide CRF receptor antagonists that can specifically and selectively block the CRF1 receptor subtype have recently been identified. Compounds such as CP 154,526 (12), NBI 27914 (129) and Antalarmin (154) inhibit CRF-stimulation of cAMP or CRF-stimulated ACTH release from cultured rat anterior pituitary cells. Furthermore, when administered peripherally, these compounds compete

for ex vivo [<sup>125</sup>I]sauvagine binding to CRF1 receptors in brain sections demonstrating their ability to cross the blood-brain-barrier. In in vivo studies peripheral administration of these compounds attenuate stress-induced elevations in plasma ACTH levels in rats demonstrating that CRF1 receptors can be blocked in the periphery. Furthermore, peripherally administered CRF1 receptor antagonists have also been demonstrated to inhibit CRF-induced seizure activity. These data clearly demonstrate that non-peptide CRF1 receptor antagonists, when administered systemically, can specifically block central CRF1 receptors and provide tools that can be used to determine the role of CRF1 receptors in various neuropsychiatric and neurodegenerative disorders. In addition, these molecules will prove useful in the discovery and development of potential orally active therapeutics for these disorders.

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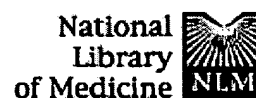
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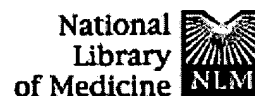
### Identification and biochemical characterization of a novel nortriterpene inhibitor of the human lymphocyte voltage-gated potassium channel, Kv1.3.

**Felix JP, Bugianesi RM, Schmalhofer WA, Borris R, Goetz MA, Hensen OD, Bao JM, Kayser F, Parsons WH, Rupprecht K, Garcia ML, Kaczorowski GJ, Slaughter RS.**

Department of Membrane Biochemistry, Merck Research Laboratories, Rahway, New Jersey 07065-0900, USA.

A novel nortriterpene, termed correolide, purified from the tree *Spachea corraea*, inhibits Kv1.3, a Shaker-type delayed rectifier potassium channel present in human T lymphocytes. Correolide inhibits 86Rb<sup>+</sup> efflux through Kv1.3 channels expressed in CHO cells (IC<sub>50</sub> 86 nM; Hill coefficient 1) and displays a defined structure-activity relationship. Potency in this assay increases with preincubation time and with time after channel opening. Correolide displays marked selectivity against numerous receptors and voltage-gated ion channels. Although correolide is most potent as a Kv1.3 inhibitor, it blocks all other members of the Kv1 family with 4-14-fold lower potency. C20-29-[3H]dihydrocorreolide (diTC) was prepared and shown to bind in a specific, saturable, and reversible fashion (K<sub>d</sub> = 11 nM) to a single class of sites in membranes prepared from CHO/Kv1.3 cells. The molecular pharmacology and stoichiometry of this binding reaction suggest that one diTC site is present per Kv1.3 channel tetramer. This site is allosterically coupled to peptide and potassium binding sites in the pore of the channel. DiTC binding to human brain synaptic membranes identifies channels composed of other Kv1 family members. Correolide depolarizes human T cells to the same extent as peptidyl inhibitors of Kv1.3, suggesting that it is a candidate for development as an immunosuppressant. Correolide is the first potent, small molecule inhibitor of Kv1 series channels to be identified from a natural product source and will be useful as a probe for studying potassium channel structure and the physiological role of such channels in target tissues of interest.

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**Potent, low-molecular-weight non-peptide inhibitors of malarial aspartyl protease plasmepsin II.****Haque TS, Skillman AG, Lee CE, Habashita H, Gluzman IY, Ewing TJ, Goldberg DE, Kuntz ID, Ellman JA.**

Department of Chemistry, University of California, Berkeley, Berkeley, California 94720, USA.

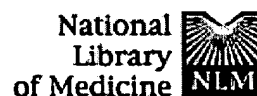
A number of single-digit nanomolar, low-molecular-weight plasmepsin II aspartyl protease inhibitors have been identified using combinatorial chemistry and structure-based design. By identifying multiple, small-molecule inhibitors using the parallel synthesis of several focused libraries, it was possible to select for compounds with desirable characteristics including enzyme specificity and minimal binding to serum proteins. The best inhibitors identified have  $K_i$ 's of 2-10 nM, molecular weights between 594 and 650 Da, between 3- and 15-fold selectivity toward plasmepsin II over cathepsin D, the most closely related human protease, good calculated log P values (2.86-4.56), and no apparent binding to human serum albumin at 1 mg/mL in an in vitro assay. These compounds represent the most potent non-peptide plasmepsin II inhibitors reported to date.

PMID: 10212129 [PubMed - indexed for MEDLINE]

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FULL-TEXT ARTICLE**N,N-dialkyl-dipeptidylamines as novel N-type calcium channel blockers.****Hu LY, Ryder TR, Rafferty MF, Cody WL, Lotarski SM, Miljanich GP, Millerman E, Rock DM, Song Y, Stoehr SJ, Taylor CP, Weber ML, Szol BG, Vartanian MG.**Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Compar  
Ann Arbor, MI 48105, USA.

Selective N-type voltage sensitive calcium channel (VSCC) blockers have shown utility in several models of stroke and pain. We are especially interest in small molecule N-type calcium channel blockers for therapeutic use. Here we report a series of N,N-dialkyl-dipeptidylamines with potent functional activity at N-type VSCCs and in vivo efficacy. The synthesis, SAR, and pharmacological evaluation of this series are discussed.

PMID: 10206559 [PubMed - indexed for MEDLINE]

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in PubMed Central**Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein.****Peterson RT, Desai BN, Hardwick JS, Schreiber SL.**

Howard Hughes Medical Institute, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA.

The FKBP12-rapamycin-associated protein (FRAP; also called RAFT1/mTOR) regulates translation initiation and entry into the cell cycle. Depriving cells of amino acids or treating them with the small molecule rapamycin inhibits FRAP and results in rapid dephosphorylation and inactivation of the translational regulators 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and p70(s6k) (the 70-kDa S6 kinase). Data published recently have led to the view that FRAP acts as a traditional mitogen-activated kinase, directly phosphorylating 4E-BP1 and p70(s6k) in response to mitogenic stimuli. We present evidence that FRAP controls 4E-BP1 and p70(s6k) phosphorylation indirectly by restraining a phosphatase. A calyculin A-sensitive phosphatase is required for the rapamycin- or amino acid deprivation-induced dephosphorylation of p70(s6k), and treatment of Jurkat T cells with rapamycin increases the activity of the protein phosphatase 2A (PP2A) toward 4E-BP1. PP2A is shown to associate with p70(s6k) but not with a mutated p70(s6k) that is resistant to rapamycin- and amino acid deprivation-mediated dephosphorylation. FRAP also is shown to phosphorylate PP2A in vitro, consistent with a model in which phosphorylation of PP2A by FRAP prevents the dephosphorylation of 4E-BP1 and p70(s6k), whereas amino acid deprivation or rapamycin treatment inhibits FRAP's ability to restrain the phosphatase.

PMID: 10200280 [PubMed - indexed for MEDLINE]

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